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APPLICATION NUMBER: *60/484,595*
FILING DATE: *July 02, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/21414*

Certified by

Jon W Dudas

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PTO/SB/16 (10-01)
Approved for use through 10/31/2002 OMB 0651-0037
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c).

Express Mail Label No. EV 286857561 US

Docket Number: 890003-3008

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☐ *Additional inventors are being named on the ____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max.)

Tissue-Engineered Blood Vessels

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ENCLOSED APPLICATION PARTS (Check all that apply)

☒ Specification *Number of Pages* **50** ☐ CD(s), Number

☒ Drawing(s) *Number of Sheets* **13** ☐ Other (specify)

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.
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FILING FEE AMOUNT ($) $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No
☐ Yes, the name of the U.S. government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE _____ DATE **July 2, 2003**

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

CERTIFICATE OF MAILING - SEPARATE PAPER -

Attorney Docket No.	:	890003-3008
Serial No.	:	To Be Assigned
Filed	:	Herewith
Applicants	:	Tranquillo et al.
Title	:	TISSUE-ENGINEERED BLOOD VESSELS

Mailed: **July 2, 2003** via "Express Mail Label # **EV 286857561 US**

I hereby certify that the above-referenced Utility Application is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to:

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TITLE OF THE INVENTION

TISSUE-ENGINEERED BLOOD VESSELS

RELATED APPLICATIONS/PATENTS & INCORPORATION BY REFERENCE

Each of the applications and patents cited in this text, as well as each document or reference cited in each of the applications and patents (including during the prosecution of each issued patent; "application cited documents"), and each of the PCT and foreign applications or patents corresponding to and/or claiming priority from any of these applications and patents, and each of the documents cited or referenced in each of the application cited documents, are hereby expressly incorporated herein by reference. More generally, documents or references are cited in this text, either in a Reference List before the numbered paragraphs, or in the text itself; and, each of these documents or references ("herein-cited references"), as well as each document or reference cited in each of the herein-cited references (including any manufacturer's specifications, instructions, etc.), is hereby expressly incorporated herein by reference. Reference is specifically made to International application nos. PCT/US00/21387, filed on August 4, 2000 (published as WO 01/110011 on February 15, 2001), and PCT/US02/04652, filed on February 14, 2002 (published as WO 02/064748 on August 22, 2002), the contents of which are incorporated herein by reference.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

This work was supported by NHLBI Grant HL60495. The government may have certain rights to the invention.

FIELD OF THE INVENTION

The present invention relates to tissue engineered blood vessel substitutes and methods of making such vessels using fibrillar biopolymeric matrices and stem cells programmed to differentiate into endothelial and smooth muscle cell lineages. Other aspects of the invention are described in or are obvious from the following disclosure, and are within the ambit of the invention.

BACKGROUND OF THE INVENTION

Surgical treatment of vascular disease has become common, creating the need for a readily available, small-diameter vascular graft. Many patients who are in need of bypass surgery do not possess sufficient veins to act as replacements for their diseased arteries. Such medical realities have propagated efforts to engineer biological replacements for such arteries. The characteristics proposed for an "ideal" tissue-engineered small diameter artery include the following: it must be biocompatible, that is, non-thrombogenic and non-immunogenic, be able to simulate the physical attributes of arteries, i.e. elasticity, contractility, compliance (viscoelasticity), adequate strength, physiological transport properties (i.e. appropriate permeability to solutes and cells), and be resistant to infection as well (Mayer, J.E. et al, 2001; Conte, M.S., 1998; Niklason, L.E., 1999; Nerem, R.M., 2000). All of these characteristics are associated with a confluent, non-activated endothelium. Moreover, these characteristics ultimately result in an acceptable wound healing response without fibrosis.

From a practical standpoint, suturability and simplicity of handling of ideal tissue-engineered blood vessels are also necessary features; and, from a commercial standpoint, the product must be fabricated by a process that scales well with quantity and the product must be able to be shipped and stored. Clearly, both the challenge and the potential of tissue engineering lie in the design of an implant that is intended to interact with host tissue. In the case of a blood vessel, the construct must provide the mechanical and hemodynamic properties critical for a vascular conduit upon implantation, and retain these properties during remodeling by the host tissue.

Weinberg and Bell pioneered the first attempt at building blood vessels by demonstrating the feasibility of creating an adventitia-like layer made from fibroblasts and collagen, a media-like layer made from smooth muscle cells (SMCs) and collagen, and an intima-like endothelial cell (EC) layer constructed into a tubular configuration. Unfortunately, in order to withstand physiological pressures, these constructs required support sleeves made from Dacron ™, a synthetic material (Weinberg, C.B. and Bell, E., 1986). Synthetic materials raise biocompatibility issues and are less desirable for use in tissue engineering applications.

There are other main approaches currently being investigated, all of which satisfy an apparent prerequisite of biocompatibility of a small diameter graft - that no permanent synthetic materials are used. One such approach is acellular, based on implanting decellularized tissues treated to enhance biocompatibility, strength, and cell adhesion/invasion leading to cellularization with host cells (Huynh, T. et al, 1999). It has yet to be elucidated whether these acellular grafts will elicit an inflammatory response in humans, and whether they will develop the host endothelial layer. Badylak and coworkers also attempted to use an implanted noncellular construct consisting of a rolled small intestinal submucosa (SIS) as a small diameter vascular graft, which serves to recruit cells from surrounding host tissue (Badylak, S. et al, 1999). However, as with other acellular studies, this study suffered from a lack of non-thrombogenic EC lining on the lumen of the graft.

Other approaches involve implantation of constructs possessing some degree of cellularity. The most recent of these is based on the concept of "self-assembly" wherein smooth muscle cells are grown to overconfluence on tissue culture plastic in medium inducing high extracellular matrix (ECM) synthesis (L'Heureux, N. et al, 1998; L'Heureux, N. et al, 2001). This leads to sheets of "neo-tissue" which are subsequently processed into multi-layer tubular form resembling the medial layer. The tube is cultured to maturity over a time span of 8 weeks. During maturation, the cells assumed a circumferential orientation and produced large amounts of ECM. While these artificial vessels could withstand impressive pressure stress, displaying rupture strengths comparable to those of native human coronary arteries, when grafted into a dog transplant model, the vessels displayed a 50% thrombosis rate after one week of implantation. This may be attributed to xenograft rejection.

Other approaches rely on a polymeric scaffold. One is based on forming a tube of a synthetic biodegradable polyglycolic acid polymer mesh and then seeding aortic SMCs and culturing it for a period of time, relying on active cell invasion or an applied pulsatile force to achieve cellularity (Shinoka, T. et al, 1998; Niklason, L.E. et al, 1999; Shinoka, T. et al, 2001; Niklason, L.E. et al, 2001). The other is based on a tube of a biopolymer formed with and compacted by tissue cells, where an appropriately applied mechanical constraint to the compaction yields circumferential alignment of fibrils and cells characteristic of the arterial

medial layer (L'Heureux, N. et al, 1993; Barocas, V.H. et al, 1998; Seliktar, D. et al, 2000). However, the constructs lacked burst strength.

The circumferential alignment is what is most attractive about a biopolymer-based tissue engineered artery. This follows from two axioms: first, that native artery function, particularly mechanical function, depends on structure, more particularly, *alignment* of the SMCs and collagen fibers in the medial layer, as much as it depends on composition. Secondly, the tissue-engineered artery should serve as a functional remodeling template, so that while providing function during the remodeling, the artificial tissue also provides a template for the alignment of the remodeled tissue.

To some extent, all of these approaches rely on the ability of cells, transplanted or autologous, to adhere to and migrate within the construct, and to remodel its composition and/or structure. This last point is key, as remodeling confers biocompatibility, in principle, by virtue of complete resorption of the initial scaffold. Accordingly, functional cell-derived ECM on the same time-scale must replace it. Remodeling also determines the ultimate mechanical, transport and biological properties. Thus, attaining the ideal tissue-engineered artery is dependent on a large number of fabrication variables that affect remodeling, including polymer type and structural characteristics, for example, density, stiffness, pore size, and, if relevant, fiber diameter. The cell type and source (i.e., fibroblasts vs. SMCs vs. EC, species, passage number, adult vs. neonatal differentiated cells, cells originating from stem cells), medium composition (serum and/or supplementing factors), nutrient availability (diffusional limitations determined by construct thickness and cell loading), mechanical stress state (static vs. cyclic, mechanical vs. hydrodynamic), autocrine factors (related to construct thickness and cell loading), and cell phenotype are all conditions which are critical for successful engineering of vascular tissue.

The approaches described above have met the various criteria of the ideal tissue-engineered artery to varying degrees. Generally, the first criterion of concern following biocompatibility is adequate mechanical, or burst, strength. While there is no standard for what the initial burst strength must be, burst pressures exceeding 2000 mm Hg have been reported at the *in vitro* stage for all approaches except for the biopolymer-based tissue-engineered artery. There have been relatively few *in vivo* studies. One published *in vivo* study using the acellular approach (chemically cross-linked submucosal collagen from small

intestine) reported 100% patency in rabbits out to 13 weeks with invasion and indications of organotypic organization of invading smooth muscle and endothelial cells (Huynh, T. et al, 1999). A sole published *in vivo* study using the self-assembly approach was limited by use of xenogeneic cells; the absence of an endothelium (to avoid hyperacute rejection) yielded low patency over the week studied (L'Heureux, N. et al, 1998).

Notably, no approach has yet resulted in all the key features of a physiological vessel, namely circumferential alignment of the smooth muscle cells and collagen fibers *and* elastin lamellae. In fact, mature elastin fibers have only been reported in the self-assembly approach, and in association with fibroblasts, not SMCs (L'Heureux, N. et al, 1998). Elasticity is critical to abolish permanent creep, and is naturally conferred by elastin lamellae. The developmental down-regulation of elastogenesis by SMCs continues to be a major hurdle. Being able to induce reversion from the synthetic phenotype, which is desirable for remodeling during cell-based construct fabrication *in vitro*, back to the contractile phenotype at implantation is another SMC related issue.

Obviously, many challenges remain. The greatest will be meeting all criteria for the ideal tissue engineered artery simultaneously. For example, high burst strength is often at the expense of a compliance mismatch, which can lead to intimal hyperplasia at the suture line (L'Heureux, N. et al, 1998). Conversely, constructs that possess physiological compliance, lack burst strength (Girton, T.S., et al, 2000). The immune response remains a challenge, with the high immunogenicity of the endothelial cell being the primary obstacle to using non-autologous cells without immunosuppression, and thus a barrier to widespread application and low-cost mass production by any approach. Finally, the ability to control the events that occur post-implantation is a major challenge, such as further construct remodeling and angiogenesis for blood supply, ultimately resulting in maintenance or enhancement of function.

OBJECT AND SUMMARY OF THE INVENTION

The demand for small-diameter vascular grafts, especially autologous ones, is extremely high. Traditionally, tissue-engineered blood vessels have used a separate endothelialization step, regardless of material composition. It has now been surprisingly

demonstrated that SMCs and ECs can be co-entrapped in fibrin-based biopolymeric constructs as a means to produce a tissue engineered blood vessel.

Thus, the present invention relates to tissue engineered blood vessel substitutes and methods of making vessels using fibrillar biopolymeric matrices with ECs and SMCs. In a preferred embodiment, the ECs and SMCs are derived from stem cells programmed to differentiate into these lineages, thereby providing a virtually unlimited source of cells. Other aspects of the invention are described in or are obvious from the following disclosure, and are within the ambit of the invention.

One aspect of the present invention is a method of producing a tissue-engineered blood vessel by entrapping at least endothelial cells (ECs) and smooth muscle cells (SMCs) in a biopolymeric matrix; and culturing the cells on the outer surface of a porous tube, wherein medium comprising a tactic factor, such as vascular endothelial growth factor (VEGF), is present inside the tube, such that a bilayered structure is formed. By this method, an endothelium (intimal layer) is formed around the tube, surrounded by a medial layer comprising SMCs.

Another embodiment is a method of providing a tissue-engineered vascular graft to a subject in need thereof, comprising entrapping at least endothelial cells (ECs) and smooth muscle cells (SMCs) in a biopolymeric matrix; culturing the cells on the outer surface of a porous tube, wherein medium comprising a tactic factor, such as vascular endothelial growth factor (VEGF), is present inside the tube, such that a bilayered structure is formed, thereby producing a tissue-engineered blood vessel; and implanting the tissue-engineered blood vessel into the subject.

Also contemplated are tissue-engineered blood vessels made by the methods of the invention, and cell-matrix structures for use as a vascular tissue comprising an a medial layer of cells and an intimal layer of cells. The blood vessels or cell matrix structure may optionally have an adventitial layer comprising fibroblasts.

A further aspect of the invention is method of culturing cells comprising endothelial cells (ECs) and smooth muscle cells (SMCs) entrapped in a biopolymeric matrix comprising the steps of growing the ECs and SMCs on the exterior surface of a porous hollow tube and providing medium comprising tactic factors axially within the interior of the porous hollow tube, such that a bilayered hollow tubular structure of cells comprising endothelial cells

(ECs) positioned interiorly and smooth muscle cells (SMCs) positioned exteriorly is formed, thereby producing a tissue-engineered blood vessel.

BRIEF DESCRIPTION OF THE DRAWINGS

The following Detailed Description, given by way of example, but not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying drawings, incorporated herein by reference, in which:

Figure 1 shows the anatomy of an artery. An artery is comprised of three layers: adventitia, media, and intima, each made up of a distinct cell population.

Figure 2 shows an immunoblot of SMC markers, α-actin, myosin, desmin, and β-actin, expressed by mesenchymal stem cells induced with platelet-derived growth factor-BB (PDGF-BB) to differentiate into smooth muscle.

Figure 3 shows smooth muscle development from multipotent adult progenitor cells (MAPCs). Cultured in the presence of PDGF-BB, MAPCs can be induced to differentiate into SMCs that express SMC cell surface markers desmin, smooth muscle actin, and smooth muscle myosin. IgG was used as a negative control.

Figure 4 shows a schematic overview of the organized tissue growth when rat SMCs are entrapped in a biocompatible polymeric matrix.

Figure 5 shows a schematic of the formation of a tubular construct of fibrin gel around a porous tubular mandrel. ECs and SMCs of the present invention will attach along the interior surface. VEGF will circulate through the axial center of the tubular construct.

Figure 6 shows Masson's Trichrome Staining of a rat aorta. Green corresponds to collagen; light red stains cell cytoplasm and muscle fibers; purple corresponds to cell nuclei; and dark red marks fibrin staining.

Figure 7 shows Verhoeff's Von Gieson Staining of a tissue-engineered medial layer formed by the process in Figure 4. Elastin fiber staining is in black; the red stain shows the presence of collagen; blue/black dots correspond to nuclei; and yellow corresponds to other tissues.

Figure 8 shows a tissue engineered blood vessel formed bythe process in Figure 4 with co-entrapment of neo-SMCs and MAPC-derived ECs using the method of Figure 5.

The micrograph depicts LDL uptake (left side of photo) relative to the plate surface (right side of photo).

Figure 9 shows a section of a vessel engineered by the methods of the invention after seven days in culture. LDL is shown in red; von Willebrand's Factor (vWF) is shown in green; and DAPI (nuclei) is shown in blue. Yellow indicates regions of overlap in LDL and vWF staining. ECs are mainly localized in the lumen after seven days of culture.

Figure 10 shows a section of a vessel engineered by the methods of the invention after three weeks in culture. LDL is shown in red; von Willebrand's Factor (vWF) is shown in green; and DAPI (nuclei) is shown in blue. Yellow indicates regions of overlap in LDL and vWF staining. ECs are exclusively located in the lumen after three weeks of culture.

Figure 11A show a cross-section of the entire construct at lower magnification immunostained for α-smooth muscle-actin (red), collagen type IV (green), and DAPI (blue). Figures 11B and C show marked organization of the cellular layers, especially of a section near the lumenal surface of the construct after 5 weeks of incubation. The vessel was immunostained for LDL (red), collagen type IV (green), and DAPI (purple; stains nuclei).

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the terms below are defined by the following meanings:

"Viscoelasticity" is a rheological parameter that describes the time-dependent deformation of a material. There are two components to the viscoelasticity: the viscosity and the elasticity. The viscosity is related to the energy dissipated during deformation and the elasticity is related to the energy stored during deformation.

"Creep" defines a slow progressive deformation of a material under constant stress.

"Patency" is defined as the ability to keep veins and arteries open.

"Vasoactivity" is the expanding and contracting of blood vessels to accommodate variations in blood flow, regulate arterial pressure, and meet the metabolic demands of the organs and body tissues.

"Lineage" refers to the delineation of a cell that has begun the natural progression from an immature cell type to one or more differentiated cell types.

"Commitment" is used to describe cells whose fate is already determined along a particular path of differentiation, at least within the bounds of an experimental assay.

"Plasticity" refers to the ability to cross lineage boundaries.

"MAPC" refers to multipotent adult progenitor cell, which is used synonymously throughout this disclosure with the terms "multipotent adult stem cell" (MASC) and "multipotent stem cell" (MPC).

"Multipotent" refers to the ability to give rise to more than one differentiated cell type.

"Pluripotent" refers to the ability to give rise to all somatic cell types found in the embryo and adult animal. However, it is noted that pluripotent cells cannot differentiate into trophectoderm cells, which are cells responsible for implantation of the blastocyst to the uterine wall.

"Totipotent" refers to the ability to give rise to all cell types, including germ cells (e.g., egg and sperm).

"Expansion" refers to the propagation or proliferation of a cell without differentiation.

"Progenitor cells" are cells produced during differentiation of a stem cell that have some, but not all, of the characteristics of their terminally differentiated progeny. Progenitor cells are committed to a specific pathway, but not to a specific cell type.

"Heterologous cells" or "heterologous tissues" are allogeneic or xenogeneic, and are harvested from compatible donors. "Autologous cells" or "autologous tissues" are harvested from the individual in which the engineered vessel is to be implanted.

"Normal" refers to an animal or subject that is not diseased, mutated or malformed, i.e., healthy animals.

"Self-renewal" refers to the ability to produce daughter stem cells that exhibit properties identical to those from which they arose. A similar term in this context is "proliferation". "Proliferation" also refers to the ability of the cell to regenerate and produce exact copies of itself, without maturing or differentiating.

"High levels of telomerase activity" can be correlated to the two-fold or higher level observed in the immortal human cell line MCF7 compared to normal cells (Soule et al. (1973) *J. Cancer Inst.* 51:1409-1416).

"Tactic factors" are factors, preferably growth factors and/or angiogenic factors, that induce the formation of a bilayered structure from cells entrapped in a biopolymeric matrix. The preferred tactic factor of the invention is VEGF.

The terms "comprises", "comprising", and the like can have the meaning ascribed to them in U.S. Patent Law and can mean "includes", "including" and the like.

Normal muscle arteries have a trilamellar structure and each of the three layers confers specific functional properties on blood vessels (Figure 1). The inner endothelial layer, or intima, is a single cell layer that prevents spontaneous blood clotting in the vessel and regulates vascular smooth muscle cell tone. The intimal layer is attached to a basement membrane, which is a thin layer of specialized ECM that can be readily identified by the presence of type IV collagen and laminin. The intermediate, or medial, layer is composed of smooth muscle cells and extracellular matrix components such as collagen, elastin, and proteoglycans. The medial layer contributes the bulk of the mechanical strength to the vessel as well as its native ability to contract or relax in response to external stimuli. The outer adventitial layer, composed primarily of fibroblasts and extracellular matrix, harbors the microscopic blood supply of the artery as well as its nerve supply. Mimicry of some or all of the properties of the three layers of a healthy artery has been the strategy of all vascular tissue engineering approaches.

Aspects and Preferred Embodiments of the Invention

The present invention provides tissue-engineered blood vessels and methods of making said vessels by mixing and co-entrapping SMCs and ECs with a fibrillar biopolymeric matrix, preferably fibrin-based. The cells contract the matrix around a nonadhesive tube, which promotes circumferential alignment of fibrils and cells. Supplementation of a tactic factor, such as the cytokine vascular endothelial growth factor (VEGF), to the culture medium in the lumen of the tube then allows organization of the entrapped cells into the intimal and medial layers present in physiological blood vessels. In a preferred embodiment, MAPCs are induced to differentiate into endothelial and smooth muscle lineages for admixing with the biopolymeric matrix.

One aspect of the present invention is a method of producing a tissue-engineered blood vessel by entrapping at least endothelial cells (ECs) and smooth muscle cells (SMCs) in a biopolymeric matrix; and culturing the cells on the outer surface of a porous tube, wherein medium comprising a tactic factor, such as vascular endothelial growth factor (VEGF), is present inside the tube, such that a bilayered structure is formed. By this method, a blood vessel is produced, comprising an endothelium (intimal layer) formed around the

tube and surrounded by a medial layer comprising SMCs. The tissue-engineered blood vessel is preferably vasoactive, implantable in a subject, and non-immunogenic to the subject in which it is implanted.

Another embodiment is a method of providing a tissue-engineered vascular graft to a subject in need thereof, comprising entrapping at least endothelial cells (ECs) and smooth muscle cells (SMCs) in a biopolymeric matrix; culturing the cells on the outer surface of a porous tube, wherein medium comprising a tactic factor, such as vascular endothelial growth factor (VEGF), is present inside the tube, such that a bilayered structure is formed, thereby producing a tissue-engineered blood vessel; and implanting the tissue-engineered blood vessel into the subject. The subject is preferably a human.

In another embodiment, the bilayered structure is coated with fibroblasts to produce a trilayered structure.

A further aspect of the invention is method of culturing cells comprising endothelial cells (ECs) and smooth muscle cells (SMCs) entrapped in a biopolymeric matrix comprising the steps of growing the ECs and SMCs on the exterior surface of a porous hollow tube and providing medium comprising tactic factors axially within the interior of the porous hollow tube, such that a bilayered hollow tubular structure of cells comprising endothelial cells (ECs) positioned interiorly and smooth muscle cells (SMCs) positioned exteriorly is formed, thereby producing a tissue-engineered blood vessel. In a preferred embodiment, the medium is flowing through the porous hollow tube. The hollow tubular structure can be surrounded by an outer casing.

In one embodiment, the ECs and/or the SMCs are derived from vascular tissue, preferably pulmonary artery, pulmonary vein, femoral artery, femoral vein, saphenous artery, saphenous vein, iliac artery, iliac vein, umbilical artery, umbilical vein, microvascular tissue, adipose, placental, and aortic tissue. Microvascular tissue is preferably derived from heart, lung, liver, kidney, brain or dermal tissue, and can be autologous or heterologous to a subject who will receive the tissue-engineered blood vessel of the invention.

In a preferred embodiment, the ECs and/or the SMCs are derived from stem cells. The stem cells can be embryonic stem (ES) cells, embryonic germ (EG) cells, multipotent adult progenitor cells (MAPCs), hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and endothelial progenitor cells (EPCs). Most preferably, the stem cells are

MAPCs. Stem cells can be derived from any appropriate tissue, and are preferably derived from bone marrow, brain, spinal cord, umbilical cord blood, liver, placenta, blood, adipose tissue, or muscle.

The ECs and SMCs (or the stem cells from which they are differentiated) can be derived from autologous or heterologous to a subject who will receive the tissue-engineered blood vessel of the invention. Preferably they are autologous and are non-immunogenic, such that an immune response is not elicited in a subject receiving a tissue-engineered blood vessel according to the invention. As such, no immunosuppression is required prior to or following engraftment of the tissue-engineered blood vessel of the invention into a subject.

The biopolymeric matrix in which the ECs and SMCs are co-entrapped can be any suitable matrix. Preferably the matrix is fibrin, fibrinogen and thrombin, laminin, collagen, fibronectin, proteoglycans, amphiphilic di-block copolymers, or amphiphilic tri-block copolymers. Even more preferably, the matrix is fibrin or fibrinogen and thrombin.

Also contemplated are tissue-engineered blood vessels made by the methods of the invention, and cell-matrix structures for use as a vascular tissue comprising an a medial layer of cells and an intimal layer of cells. The blood vessels or cell matrix structure may optionally have an adventitial layer comprising fibroblasts. The structure can be in culture, and the intimal layer is preferably in contact with a medium comprising a tactic factor, preferably VEGF or another angiogenic factor. In a preferred embodiment, the structure further comprises a basement membrane, and a lumen. The layers of the vascular tissue can be identified by any method known to the skilled artisan, such as immunostaining, immunoblotting, magnetic beads, flow cytometry, microarray analysis, or RT-PCR. The vascular tissue is preferably vasoactive and can be an artery or a vein.

The cell-matrix structure can further comprise a porous tube, around which the intimal and medial layers of cells are positioned concentrically. The cell-matrix structure can also comprise an outer casing surrounding the cell-matrix structure.

The tube used to culture the cell-matrix structure is preferably comprised of porous plastic, and even more preferably, the porous plastic is polyethylene. In a preferred embodiment, the tube further is coated with agarose or some other permeable substance that renders it non-adhesive to the matrix. The mold to form a tubular fibrin gel for co-culturing cells is composed of a tubular porous polyethylene mandrel (70 μm pore size),.rubber

stoppers, and a tubular outer casing as shown in Figure 5. Rubber stoppers are placed on the ends of the porous mandrel. The mandrel with stoppers is dipped into a 2% agarose solution to provide a nonstick surface on the outer surface of the polyethylene mandrel. The coated mandrel with stoppers is then placed into an outer casing made of plastic or glass. A needle is inserted between the rubber stopper and the outer casing at one end to allow air to escape as the space is filled with solution. Once the fibrinogen solution, cell suspension, and thrombin solution are mixed, they are injected between the outer casing and the porous mandrel using a needle and syringe. The needles are removed and the solution is then allowed to gel at 37°C in an incubator. A plunger is used to gently push the rubber stoppers, porous mandrel, and fibrin gel out of the outer casing and into a container containing culture medium. See Figure 4 for a schematic representation of the co-entrapment and organization process.

Biomaterials of the Present Invention

Many different biomaterials have been used as scaffolds for tissue repair. These biomaterials all have selected uses for which they are well suited, but a common feature of scaffolds that are effectively replaced (i.e. resorbed/degraded) by host tissues is the ability to support new blood vessel growth, that is, angiogenesis. Angiogenesis is necessary for the nutrition of cells that will populate a tissue scaffold, elimination of waste products of these cells, defense against infectious agents that may attempt to populate the biomaterial, and for the removal of degradation products of resorbable scaffold materials. Both angiogenesis and the *in vitro* behavior of endothelial cells are complex phenomena, with multiple progressive steps toward the endpoint of new blood vessel formation. Angiogenesis requires a substrate to which endothelial cells can adhere, migrate upon, proliferate within, and then differentiate into a mature endothelial cell phenotype. The physical characteristics, as well as the composition of the material, must be suitable for endothelial cell adherence. Under physiological conditions, the extracellular matrix of normal body tissues plays a crucial role in all of these events as they relate to angiogenesis.

An acellular resorbable scaffold material derived from the small intestinal submucosa (SIS) has been shown to be rapidly resorbed, support early and abundant new blood vessel growth, and serve as a template for the constructive remodeling of several body tissues including musculoskeletal structures, skin, body wall, dura mater, urinary bladder, and blood

vessels (Badylak, S. et al, 1999). Other biomaterials suitable for use as vascular scaffolds include fibrillar collagen, laminin, fibronectin, proteoglycans, glycosaminoglycans, and fibrin. Matrigel™ is one such example of a basement membrane matrix. Matrigel™ matrix is composed of laminin, collagen IV, nidogen/enactin, and proteoglycans, however the composition is heterogeneous and not all components of the matrix are known. This drawback often makes critical interpretation of results difficult. Other ECM-based gels, containing known components, can be advantageously used, such as collagen gels, laminin gels, and fibrin/fibrinogen gels. A preferred embodiment of the present invention utilizes fibrin-based polymeric matrices, including the monomeric precursor, fibrinogen, in combination with thrombin to cleave fibrinogen into fibrinopeptides that self-assemble into fibrin fibrils ("fibrin").

Other scaffolds that are envisioned by the instant invention include amphiphilic block copolymers (Nardin, C. and Meier, W., 2002). These molecules consist of at least two parts with different solubilities, causing their self-assembly into superstructures in the sub-micrometer range, with cores consisting of their insoluble parts, surrounded by their soluble parts. This self-organization of block copolymers is based on the same underlying principles as for typical low molecular weight amphiphiles, such as surfactants or lipids in water. The high diversity of block copolymer chemistry allows for variation of the chemical constitution, the length and structure of different blocks, and the molecular architecture of the whole polymer. The equilibrium shape of amphiphilic aggregates is related to the molecular geometry of the underlying molecules. Manipulation of the shape and length of the hydrophilic blocks can cause a transition from spherical to worm-like micelles, and finally to vesicular structures. Such block copolymers include poly(ethyleneoxide)-poly(butadiene), or PEO-PB diblock copolymer, which forms giant worm-like micelles of dimensions similar to fibrin fibrils at low concentrations in water and exhibit viscoelastic properties in water (Won, Y.Y. et al, 1999) similar to a fibrin gel.

Other materials can include the siloxanes, such as poly[(aminopropyl)siloxane] (poly-APS) as a coupling agent to bond inorganic to organic materials. Poly-APS structures are attractive because they form extended oligomeric structures consisting of linear, cyclic, and cross-linked Si—O—Si polymeric networks. Additionally, poly-APS structures are amphiphilic, consisting of inorganic silanol and organic aminopropyl groups. Recent studies

indicate that a poly-APS film doped with potassium ions forms a structure that self-assembles, has built-in error correction, forms well-defined fibrous structures, and in high-yields. Also, these microfibers can be easily disassembled by dissolution in an aqueous environment at room temperature (Celio, H. et al, 2003).

Sources of Endothelial, Smooth Muscle, and Fibroblast Cells

Endothelial cells can be harvested from any vascular source. Typical sources of ECs include aorta, saphenous vein, femoral artery, iliac artery, iliac vein, pulmonary artery, pulmonary vein, placenta, adipose vascular and microvascular tissue, and umbilical vein and artery. Microvascular sources are also contemplated by the present invention, such as, but not limited to, heart coronary tissue, liver and sinusoidal tissues, lung, kidney, brain, and dermis-derived microvascular tissue sources. Primary cell lines such as human microvascular endothelial cells (HMECs) and human umbilical vein endothelial cells (HUVECs) are commonly used sources for endothelial cells, and grow well under certain conditions, in tissue culture. Vessel-derived endothelial cells can be isolated by cannulation (i.e. "gut cleaners") and incubation of vascular tissues with collagenases or matrix metalloproteinases. Isolation of ECs are exemplified by work by Jaffe and coworkers (Jaffe, E.A., et al. 1973). The identity of endothelial cells can be confirmed by their production of von Willebrand factor (vWF), and uptake of acylated low-density lipoprotein (acLDL).

Vascular smooth muscle cells also can be harvested from vascular sources, similar to endothelial cells. A typical source, like endothelial cells, is umbilical vein and artery, but can also include aorta, saphenous vein, femoral artery, iliac artery, iliac vein, pulmonary artery, and pulmonary vein, as well as heart, liver, lung, kidney, brain, and dermis-derived microvascular tissues. Harvesting and isolation of SMCs are described in Ross, 1971. Vascular smooth muscle cells can be advantageously identified by the presence of α-actin, desmin, and smooth muscle myosin. Antibodies against these SMC-specific cellular markers are well known in the art and are commercially available.

Fibroblasts can be harvested from bone marrow, lung, embryo, adipose tissue, subcutaneous connective tissue, areolar connective tissue, kidney, skin, and brain. Fibroblasts are precursors of many different types of connective tissue and can differentiate into osteoblasts of bone, adipocytes of fat, and chondrocytes in cartilage. Fibroblasts can be advantageously identified by the presence of prolyl-4-hydroxylase B and type I procollagen

(Janin, A. et al, 1990). Fibroblasts can also be obtained by differentiation of stem cells such as MAPCs.

Monitoring the progress of endothelial and smooth muscle cell differentiation can involve, for example, screening for expression of genetic markers of EC and SMC differentiation. Genetic markers of ECs are well known in the art, and include vWF, acLDL uptake, β-catenin, γ-catenin, connexin-40, connexin 43, ZO-1, c-Kit, CD31 (PECAM-1), CD62P, CD62L, CD62E, αVβ3, αvβ5, E-cadherin, VE-cadherin, Flt1, Flk1 (VEGF-R2), Tie/Tek, VCAM-1, and CD105 (Jaffe, E.A. et al, 1974; Stein, O. and Stein, Y., 1976; Tao, Y.S. et al, 1996; Lim, M.J. et al, 2001; Van Rijen, H. et al, 1997; Watson, P.M. et al, 1991; Buzby, J.S. et al, 1994; Albelda, S.M. et al, 1990; Johnston, G.I. et al, 1989; Friedlander, M. et al, 1995; Bevilacqua, M.P. et al, 1989; Buhrer, C. et al, 1990; Lawler, J. and Hynes, R.O., 1989; Bavisotto, L.M. et al, 1990; Breviario, F. et al, 1995; Waltenburger, J. et al, 1994; Chiang, M.K. and Flanagan, J.G., 1995; Partanen, J. et al, 1992; Dumont, D.J. et al, 1993; Osborn, L. et al, 1989; Gougos, A. and Letarte, M., 1990; reviewed in Bachetti, T. and Morbidelli, L., 2000). Genetic markers of SMCs are also known, and can include, but are not limited to, desmin, smooth muscle α-actin, calponin, smoothelin, and smooth muscle myosin heavy chain and light chains (Gabbiani, G. et al, 1981; Birukov, K.G. et al, 1991; Ratajska, A. et al, 2001; reviewed in Sobue, K. et al, 1999). It will be apparent to those skilled in the art that not all markers listed above are restricted to ECs or SMCs.

The present invention contemplates the use of autologous or isogeneic vascular cells harvested from patients undergoing tissue grafting of the engineered blood vessels described herein. Another source is vascular cells harvested from compatible donors (i.e., heterologous cells). It will be apparent to those skilled in the art that use of heterologous cells may require immunosuppression of the subject in need of a vascular graft to prevent rejection of foreign cells. Immunosuppression of the subject can be achieved using pharmacological agents such as, but not limited to, cyclosporine, tacrolimus, rapamycin, glucocorticoids such as prednisone and prednisolone, azathioprine, mycophenolate mofetil, methotrexate, cyclophosphamide, monoclonal antibodies such as muromonab-CD3, and anti-thymocyte globulin antibodies (Goodman and Gilman's The Pharmacologial Basis of Therapeutics, 9th Edition). A preferred source of cells that can be used advantageously to differentiate and proliferate into vascular tissue is stem cells. In a preferred embodiment of the instant

invention, stem cells are used to cellularize and populate tissue-engineered blood vessels. In a particularly preferred embodiment, the stem cells are autologous, eliminating the need for immunosuppression.

Stem Cells Used in the Present Invention

"Stem cells", as used in the present invention, are defined as cells that have extensive proliferation potential, differentiate into several cell lineages, and repopulate tissues upon transplantation (Donovan, P.J. and Gearhart, J., 2001; Krause, D.S. et al, 2001).

Stem cells that can be used in the present invention include MAPCs, or multipotent adult progenitor cells (Jiang, Y. et al, 2002). MAPCs are characterized as cells that constitutively express Oct-4 and high levels of telomerase and are negative for CD44 and MHC class I and class II expression. MAPCs derived from human, mouse, rat or other mammals appear to be the only normal, non-malignant, somatic cell (i.e., non-germ cell) known to date to express very high levels of telomerase even in late passage cells. The telomeres are extended in MAPCs and they are karyotypically normal. Because MAPCs injected into a mammal can migrate to and assimilate within multiple organs, MAPCs are self-renewing stem cells. As such, they have utility in the repopulation of organs, either in a self-renewing state or in a differentiated state compatible with the organ of interest. They have the capacity to replace cell types that could have been damaged, died, or otherwise might have an abnormal function because of genetic or acquired disease.

Human MAPCs are described in PCT/US00/21387 (published as WO 01/11011) and PCT/US02/04652 (published as WO 02/064748). Human MAPCs exhibit the following markers: CD10, CD13, CD49b, CD49e, CDw90, Flk1, EGF-R, TGF-R1, TGF-R2, BMP-R1A, PDGF-R1a, PDGF-R1b, gp130, LIF-R, activin-R1, activin-R2, CD49c, CD49d, CD29, hTRT, TRF1, Oct3/4, Rex-1, Rox-1, BMP1, BMP5, VEGF, HGF, KGF, MCP1, SSEA-4, Sox-2, Sox-9, Sox-11, PDGF-R1α, Dlx4, MSX1, PDX1, Hoxa4, Hoxa5, Hoxa9, β2-microglobulin^{low}, Muc18^{low}, CD44-E^{low}, Flt1^{low}, $\alpha_v B_5{}^{low}$, AC133^{low}, albumin^{low}, CK18^{low}, CK19^{low}, CYP1B1^{low}. When grown at higher confluence, these cells can express elevated levels of β2-microglobulin, Muc18 and CD44-E.

MAPCs have been identified in other mammals. Murine MAPCs are described in PCT/US00/21387 (published as WO 01/11011) and PCT/US02/04652 (published as WO 02/064748). Murine MAPCs exhibit the following markers: CD13+, Flk1, Thy1^{low}, Sca1^{low},

SSEA1^{low}, Oct3/4 and Rex1 ^{low}. They are negative for CD45, GlyA, CD44, MHC-I, MHC-II, cKit, and endoglin. Murine MAPCs can also be characterized by the expression of CD10, CD13, CD49b, CD49e, CDw90, EGF-R, TGF-R1, TGF-R2, BMP-R1A, PDGF-R1a, PDGF-R1b, gp130, LIF-R, activin-R1, activin-R2, CD49c, CD49d, CD29, hTRT, TRF1, Rox-1, BMP1, BMP5, VEGF, HGF, KGF, MCP1, SSEA-4, Sox-2, Sox-9, Sox-11, PDGF-R1∝, Dlx4, MSX1, PDX1, Hoxa4, Hoxa5, and Hoxa9.

Stem cells used in the present invention can also include embryonic stem cells (Lebkowski, J.S. et al, 2001). The quintessential stem cell is the embryonic stem (ES) cell, as it has unlimited self-renewal and pluripotent differentiation potential (Thomson, J. *et al.* 1995; Thomson, J.A. *et al.* 1998; Shamblott, M. *et al.* 1998; Williams, R.L. *et al.* 1988; Orkin, S. 1998; Reubinoff, B.E., *et al.* 2000). These cells are derived from the inner cell mass (ICM) of the pre-implantation blastocyst (Thomson, J. *et al.* 1995; Thomson, J.A. *et al.* 1998; Martin, G.R. 1981), or can be derived from the primordial germ cells from a post-implantation embryo (embryonal germ cells or EG cells). ES and/or EG cells have been derived from multiple species, including mouse, rat, rabbit, sheep, goat, pig and, more recently, from human and non-human primates (U.S. Patent Nos. 5,843,780 and 6,200,806).

When introduced into mouse blastocysts, ES cells can contribute to all tissues of the mouse (animal) (Orkin, S. 1998). Murine ES cells are therefore known to be pluripotent. When transplanted in post-natal animals, ES and EG cells generate teratomas, which again demonstrates their multipotency. ES (and EG) cells can be identified by positive staining with the antibodies to stage-specific embryonic antigens (SSEA) 1 and 4. At the molecular level, ES and EG cells express a number of transcription factors highly specific for these undifferentiated cells. These include Oct-4 and Rex-1, and leukemia inhibitory factor receptor (LIF-R). The transcription factors Sox-2 and Rox-1 are expressed in both ES and non-ES cells. Oct-4 is expressed in the pre-gastrulation embryo, early cleavage stage embryo, cells of the inner cell mass of the blastocyst, and embryonic carcinoma (EC) cells. In the adult animal, Oct-4 is only found in germ cells.

Oct-4, in combination with Rox-1, causes transcriptional activation of the Zn-finger protein Rex-1, and is also required for maintaining ES in an undifferentiated state. The Oct-4 gene is down regulated when cells are induced to differentiate *in vitro.* Several studies have shown that Oct-4 is required for maintaining the undifferentiated phenotype of ES cells, and

that it plays a major role in determining early steps in embryogenesis and differentiation (Pesce, M. and Scholer, H.R., 2000). Sox-2 is required with Oct-4 to retain the undifferentiated state of ES/EC and to maintain murine, but not human, ES cells (Wood, H.B. and Episkopou, V., 1999). Another hallmark of ES cells is the presence of high levels of telomerase, which provides these cells with an unlimited self-renewal potential *in vitro*.

Stem cells that can be used in the present invention also include those known in the art that have been identified in organs or tissues. The best characterized is the hematopoietic stem cell (HSC). This mesoderm-derived cell has been purified based on cell surface markers and functional characteristics. The HSC, isolated from bone marrow (BM), blood, cord blood, fetal liver and yolk sac, is the progenitor cell that generates blood cells, or following transplantation, reinitiates multiple hematopoietic lineages and can reinitiate hematopoiesis for the life of a recipient. (See Fei, R., *et al.*, U.S. Patent No. 5,635,387; McGlave, *et al.*, U.S. Patent No. 5,460,964; Simmons, P., *et al.*, U.S. Patent No. 5,677,136; Tsukamoto, *et al.*, U.S. Patent No. 5,750,397; Schwartz, *et al.*, U.S. Patent No. 5,759,793; DiGuisto, *et al.*, U.S. Patent No. 5,681,599; Tsukamoto, *et al.*, U.S. Patent No. 5,716,827; Hill, B., *et al.* 1996.) When transplanted into lethally irradiated animals or humans, HSCs can repopulate the erythroid, neutrophil-macrophage, megakaryocyte and lymphoid hematopoietic cell pool. *In vitro,* HSCs can be induced to undergo at least some self-renewing cell divisions and can be induced to differentiate to the same lineages observed *in vivo*. Similarly, HSCs may retain plasticity capable of regenerating multiple cell types in non-hematopoietic tissues, including endothelial cells. It is thought that HSCs and endothelial progenitor cells (EPCs) arise from a common progenitor, known as the "hemangioblast" (Choi, K., 2002). These early precursors are thought to originate from the blood islands of the yolk sac during early development (embryonic day 7.5).

Recent evidence suggests that a novel "side population" of CD34- stem cells, which are potent HSCs that give rise to all blood cell lineages, are capable of contributing to regeneration of cardiac muscle and vascular endothelial cells (Jackson, K.A. et al, 2001). Lethally irradiated mice were transplanted with a *lacZ*-expressing "side population" of HSCs, followed by occlusion and reperfusion of the left anterior descending coronary artery. HSCs were able to repopulate vessel structures, demonstrating that these cells or their progeny had migrated to the injured heart via circulatory system, localized to newly forming vessels, and

integrated into the surface lining as differentiated ECs, as detected by the presence of β-galactosidase, the *lacZ* gene product, and EC markers ICAM-2, VE-cadherin, von Willebrand factor, factor VIII, and the VEGF receptors Flk-1 and Flt-1 (Jackson, K.A. et al, 2001).

Yet another stem cell is the mesenchymal stem cell (MSC), initially described by Fridenshtein (1982). MSCs, originally derived from the embryonal mesoderm and also isolated from adult BM, can differentiate to form muscle, bone, cartilage, fat, marrow stroma, and tendon (Jiang, Y. et al, 2002). During embryogenesis, the mesoderm develops into limb-bud mesoderm, tissue that generates bone, cartilage, fat, skeletal muscle and possibly endothelium. Mesoderm also differentiates to visceral mesoderm, which can give rise to cardiac muscle, smooth muscle, or blood islands consisting of endothelial and hematopoietic progenitor cells. Primitive mesoderm or MSCs, therefore, could provide a source for a number of cell and tissue types. A number of MSCs have been isolated. (See, for example, Caplan, A., *et al.*, U.S. Patent No. 5,486,359; Young, H., *et al.*, U.S. Patent No. 5,827,735; Caplan, A., *et al.*, U.S. Patent No. 5,811,094; Bruder, S., *et al.*, U.S. Patent No. 5,736,396; Caplan, A., *et al.*, U.S. Patent No. 5,837,539; Masinovsky, B., U.S. Patent No. 5,837,670; Pittenger, M., U.S. Patent No. 5,827,740; Jaiswal, N., *et al.*, 1997; Cassiede P., *et al.*, 1996; Johnstone, B., *et al.*, 1998; Yoo, *et al.*, 1998; Gronthos, S., 1994).

Of the many MSCs that have been described, most have demonstrated limited differentiation to form cells generally considered to be of mesenchymal origin. To date, the most multipotent MSC reported is the cell isolated by Pittenger, *et al.*, which expresses the $SH2^+$ $SH4^+$ $CD29^+$ $CD44^+$ $CD71^+$ $CD90^+$ $CD106^+$ $CD120a^+$ $CD124^-$ CD 14^- $CD34^-$ $CD45^-$ phenotype. This cell is capable of differentiating into a number of cell types of mesenchymal origin (Pittenger, *et al.*, 1999). MSCs can also differentiate into endodermal and ectodermal, including neural, lineages (reviewed in Minguell, J.J. et al, 2001). Most, if not all, vessels develop from an endothelial tube that subsequently acquires a coating formed by vascular smooth muscle cells (vSMC), which in turn develop from an undifferentiated perivascular mesenchymal progenitor (Hellstrom, M. et al, 1999). MSCs are thought to migrate out of the bone marrow into specific tissues, where they in turn differentiate into multiple lineages depending on the cellular microenvironment.

Endothelial progenitor cells (EPCs) are primarily responsible for differentiation into vascular tissues and can be found in peripheral blood and bone marrow, as well as sites of physiological and pathological neovascularization (Asahara, T. et al, 1997). As described above, EPCs are generated in close association with the developing vascular system. In the blood islands of the yolk sac, where the earliest EPCs appear, both HSC and EPC lineages arise almost simultaneously from extraembryonic mesoderm, forming structures in which primitive erythrocytes are surrounded by a layer of angioblasts that give rise to differentiated ECs (Kubo, H. and Alitalo, K., 2003). It is thought that the transcription factor Tal1/SCL (T-cell acute leukemia/stem cell leukemia) regulates the choice of cell fate in early development into EC, HSC, and SMC lineages (Ema, M. et al, 2003). Transplantation of EPCs can successfully enhance vascular development by *in situ* differentiation and proliferation within ischemic organs (Kalka, C. et al, 2000).

Evidence that EPCs reside in circulating blood and bone marrow is increasing (Lin, Y. et al, 2000; Shi, Q. et al, 1998). vSMCs were previously thought to be recruited via endothelial-derived signals (Hirschi, K.K. et al, 1998; Hirschi, K.K. et al, 1999) from local mesenchyme in the region of neovascularization, or recruited along with ECs from pre-existing vessels to form the surrounding vessel wall as new vessels sprout (Lindahl, P. et al, 1998). However, blood circulation also contains an SMC progenitor population (Shimizu, K. et al, 2001). Yamashita and coworkers observed that ES cells expressing the VEGF receptor Flk-1, which is expressed early in vascular development by hemangioblasts, as well as mature ECs, give rise to vSMCs *in vivo* and *in vitro* (Yamashita, J. et al, 2000). These studies suggest that stem cells identified in adult tissues as EPCs may serve as SMC precursors as well. Notably, primary cell lines such as HUVECs also retain the ability to differentiate from ECs to SMCs in the absence of fibroblast growth factor (FGF; Ishisaki, A. et al, 2003)

There have been few reports of neural stem cells (NSCs) and their purported differentiation into ECs and SMCs, however recent studies of the cells and genes expressed in the embryonic neural crests suggests there may be similarities between neural crest and endothelial cell development (Gammill, L.S., and Bonner-Fraser, M., 2002). The cells of the neural crest give rise to almost all of the peripheral nervous system, including pigment cells of the skin (Morrison, S.J. et al, 1999). In the head, many of the neural crest cells will

differentiate into cartilage, bone, and other connective tissues, which, elsewhere in the body, arise from the mesoderm. In this instance, differentiation of cells arising from the neural crest runs counter to the general scheme in which the three germ layers give rise to cells in the three corresponding concentric layers of the adult body (ectoderm, mesoderm, endoderm). Eight genes isolated from a newly formed neural crest have been previously implicated in endothelial cell development, such as factors involved in VEGF production and signaling (ORP150 and neuropilin 2a1), as well as proteins important for EC migration, such as laminin $\alpha 5$ and $\gamma 1$. Although this comparison is novel, mechanistically, both EC and neural crest cells migrate and invade tissues as they perform their embryonic functions.

In addition, a report from Oishi and coworkers determined that multipotent neural stem cells isolated from rat embryonic day 14 or mouse embryonic day 12 cortex gave rise to both endothelial cells and smooth muscle cells. Reconstituted collagen gel fibers of NSC-derived SMCs caused contractions in response to typical contractile agonists (Oishi, K. et al, 2002). Moreover, NSCs subcultured into collagen gel formed endothelial tube-like structures (Kawakita, E. et al, 2002). This suggests that blood vessel cells in the head may be at least in part derived from NSCs.

Culture Conditions of the Present Invention

Initially, cells used in the present invention are maintained and allowed to expand in culture medium that is well established in the art and commercially available from the American Type Culture Collection (ATCC). Such media include, but are not limited to, Dulbecco's Modified Eagle's Medium® (DMEM), DMEM F12 medium®, Eagle's Minimum Essential Medium®, F-12K medium®, Iscove's Modified Dulbecco's Medium®, RPMI-1640 medium®. It is within the skill of one in the art to modify or modulate concentrations of media and media supplements as necessary for the cells used. It will also be apparent that many media are available as a low-glucose formulation, with or without sodium pyruvate.

Also contemplated is supplementation of cell culture medium with mammalian sera. Sera often contain cellular factors and components that are necessary for viability and expansion. Examples of sera include fetal bovine serum (FBS), bovine serum (BS), calf serum (CS), fetal calf serum (FCS), newborn calf serum (NCS), goat serum (GS), horse serum (HS), human serum, chicken serum, porcine serum, sheep serum, rabbit serum, serum

replacements, and bovine embryonic fluid. It is understood that sera can be heat-inactivated at 55-65°C if deemed necessary to inactivate components of the complement cascade.

Additional supplements can also be used advantageously to supply the cells with the necessary trace elements for optimal growth and expansion. Such supplements include insulin, transferrin, sodium selenium and combinations thereof. These components can be included in a salt solution such as, but not limited to Hanks' Balanced Salt Solution® (HBSS), Earle's Salt Solution®, antioxidant supplements, MCDB-201® supplements, phosphate buffered saline (PBS), ascorbic acid and ascorbic acid-2-phosphate, as well as additional amino acids. Many cell culture media already contain amino acids, however some require supplementation prior to culturing cells. Such amino acids include, but are not limited to, L-alanine, L-arginine, L-aspartic acid, L-asparagine, L-cysteine, L-cystine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine. It is well within the skill of one in the art to determine the proper concentrations of these supplements.

Antibiotics are also typically used in cell culture to mitigate bacterial, mycoplasmal, and fungal contamination. Typically, antibiotics or anti-mycotic compounds used are mixtures of penicillin/streptomycin, but can also include, but are not limited to amphotericin (Fungizone®), ampicillin, gentamicin, bleomycin, hygromycin, kanamycin, mitomycin, mycophenolic acid, nalidixic acid, neomycin, nystatin, paromomycin, polymyxin, puromycin, rifampicin, spectinomycin, tetracycline, tylosin, and zeocin. Antibiotic and antimycotic additives can be of some concern, depending on the type of work being performed. One possible situation that can arise is an antibiotic-containing media wherein bacteria are still present in the culture, but the action of the antibiotic performs a bacteriostatic rather than bacteriocidal mechanism. Also, antibiotics can interfere with the metabolism of some cell types.

Hormones can also be advantageously used in cell culture and include, but are not limited to D-aldosterone, diethylstilbestrol (DES), dexamethasone, β-estradiol, hydrocortisone, insulin, prolactin, progesterone, somatostatin/human growth hormone (HGH), thyrotropin, thyroxine, and L-thyronine. A preferred embodiment uses dexamethasone.

Lipids and lipid carriers can also be used to supplement cell culture media, depending on the type of cell and the fate of the differentiated cell. Such lipids and carriers can include, but are not limited to cyclodextrin (α, β, γ), cholesterol, linoleic acid conjugated to albumin, linoleic acid and oleic acid conjugated to albumin, unconjugated linoleic acid, linoleic-oleic-arachidonic acid conjugated to albumin, oleic acid unconjugated and conjugated to albumin, among others. A preferred embodiment uses linoleic acid conjugated to albumin.

Also contemplated is the use of feeder cell layers. Feeder cells are used to support the growth of fastidious cultured cells, including stem cells. Feeder cells are normal cells that have been inactivated by γ-irradiation. In culture, the feeder layer serves as a basal layer for other cells and supplies important cellular factors without further growth or division of their own (Lim, J.W. and Bodnar, A., 2002). Examples of feeder layer cells are typically human diploid lung cells, mouse embryonic fibroblasts, Swiss mouse embryonic fibroblasts, but can be any post-mitotic cell that is capable of supplying cellular components and factors that are advantageous in allowing optimal growth, viability, and expansion of stem cells. In many cases, feeder cell layers are not necessary to keep the stem cells in an undifferentiated, proliferative state, as leukemia inhibitory factor (LIF) has anti-differentiation properties. Often, supplementation of a defined concentration of LIF is all that is necessary to maintain stem cells in an undifferentiated state.

Cells in culture can be maintained either in suspension or attached to a solid support, such as extracellular matrix components. Stem cells often require additional factors that encourage their attachment to a solid support, such as type I, type II, and type IV collagen, concanavalin A, chondroitin sulfate, fibronectin, "superfibronectin" and fibronectin-like polymers, gelatin, laminin, poly-D and poly-L-lysine, thrombospondin, and vitronectin. Preferred embodiments utilize fibronectin.

The maintenance conditions of stem cells can also contain cellular factors that allow stem cells, such as MAPCs, to remain in an undifferentiated form. Specifically, these cellular factors or components allow the stem cells to constitutively express Oct 4, maintain high levels of telomerase, and remain negative for CD44, MHC class I and MHC class II expression. It is advantageous under conditions where the cell must remain in an undifferentiated state of self-renewal for the medium to contain epidermal growth factor (EGF), platelet derived growth factor (PDGF), leukemia inhibitory factor (LIF), and

combinations thereof. It is apparent to those skilled in the art that supplements that allow the cell to self-renew but not differentiate must be removed from the culture medium prior to differentiation.

Differentiation to ECs can occur when VEGF or other angiogenic factors are added to the culture medium. Other differentiation factors can be similarly employed to induce stem cells to become SMCs, such as PDGF-BB. Angiogenic factors include, but are not limited to, adrenomedullin, acidic fibroblast growth factor (aFGF), angiogenin, angiotensin-1 and –2, betacellulin, basic fibroblast growth factor (bFGF), corpus luteum angiogenic factor (CLAF), endothelial-cell derived growth factor (ECDGF), Factor X and Xa, HB-EGF, PD-ECGF, PDGF, angiomodulin, angiotropin, angiopoetin-1, prostaglandin E1 and E2, steroids, heparin, 1-butyryl-glycerol, nicotinic amide, and tumor necrosis factor α. Preferred embodiments of the invention utilize VEGF as a differentiation factor for ECs, and PDGF-BB for SMCs.

Stem cell lines and other fastidious cells like ECs and SMCs often benefit from co-culturing with another cell type. Such co-culturing methods arise from the observation that certain cells can supply yet-unidentified cellular factors that allow the stem cell to differentiate into a specific lineage or cell type. These cellular factors can also induce expression of cell-surface receptors, some of which can be readily identified by monoclonal antibodies. Generally, cells for co-culturing are selected based on the type of lineage one skilled in the art wishes to induce, and it is within the capabilities of the skilled artisan to select the appropriate cells for co-culture.

Methods of identifying and subsequently separating differentiated cells from their undifferentiated counterparts can be carried out by methods well known in the art. Cells that have been induced to differentiate can be identified by selectively culturing cells under conditions whereby differentiated cells outnumber undifferentiated cells. Similarly, differentiated cells can be identified by morphological changes and characteristics that are not present on their undifferentiated counterparts, such as cell size, the number of cellular processes (i.e. formation of dendrites and/or branches), and the complexity of intracellular organelle distribution. Also contemplated are methods of identifying differentiated cells by their expression of specific cell-surface markers such as cellular receptors and transmembrane proteins. Monoclonal antibodies against these cell-surface markers can be used to identify differentiated cells. Detection of these cells can be achieved through

fluorescence activated cell sorting (FACS), and enzyme-linked immunosorbent assay (ELISA). From the standpoint of transcriptional upregulation of specific genes, differentiated cells often display levels of gene expression that are different from undifferentiated cells. Reverse-transcription polymerase chain reaction (RT-PCR) can also be used to monitor changes in gene expression in response to differentiation. In addition, whole genome analysis using microarray technology can be used to identify differentiated cells.

Accordingly, once differentiated cells are identified, they can be separated from their undifferentiated counterparts, if necessary. The methods of identification detailed above also provide methods of separation, such as FACS, preferential cell culture methods, ELISA, magnetic beads, and combinations thereof. A preferred embodiment of the invention envisions the use of FACS to identify and separate cells based on cell-surface antigen expression.

The present invention is additionally described by way of the following illustrative, non-limiting Examples that provide a better understanding of the present invention and of its many advantages.

EXAMPLES

Example 1: Culture And Differentiation Conditions For MAPCs

MAPC Isolation and Culture Conditions

Bone marrow (BM) was obtained from 55 healthy volunteers donors (age 2-50 years) after informed consent using guidelines from the University of Minnesota Committee on the use of Human Subject in Research. MAPCs were generated as previously described (Furcht *et al.*). Briefly, BM mononuclear cells (BMMNC) were depleted of $CD45^+$ and glycophorin-A^+ cells using micromagnetic beads (Miltenyii Biotec, Sunnyvale, CA). $CD45^-/GlyA^-$ cells ($5x10^3$ cells) diluted in 200μL expansion medium comprising 58% low-glucose Dulbecco's minimal essential medium, low-glucose formulation (DMEM-LG) (Gibco-BRL, Grand Island, NY), 40% MCDB-201 (Sigma Chemical Co, St Louis, MO), supplemented with 1X insulin-transferrin-selenium (ITS), 1X linoleic-acid bovine serum albumin (LA-BSA), 10^{-8}M Dexamethasone, 10^{-4}M ascorbic acid 2-phosphate (all from Sigma), 100U penicillin and 1,000U streptomycin (Gibco) and 10% FCS (Hyclone Laboratories, Logan, UT) with 10

ng/ml of epidermal growth factor (EGF; Sigma) and 10 ng/ml PDGF-BB (R&D Systems, Minneapolis, MN) were plated in wells of 96 well plates that had been coated with 5ng/ml of fibronectin (FN; Sigma). Medium was exchanged every 4-6 days. Once wells were >40-50% confluent, adherent cells were detached with 0.25% trypsin-EDTA (Sigma) and replated at 1:4 dilution in bigger culture vessels, again coated with 5 ng/ml fibronectin and MAPC expansion medium to maintain cell densities between 2 and $8x10^3$ cells/ cm^2.

Differentiation Conditions and Characterization

To induce differentiation to ECs, MAPC were replated at 1-2 x 10^4 cells/cm^2 in FN coated culture vessels or chamber slides in 60% low-glucose Dulbecco's minimal essential medium (DMEM-LG) (Gibco-BRL, Grand Island, NY), 40% MCDB-201 (Sigma Chemical Co, St Louis, MO), supplemented with 1X insulin-transferrin-selenium (ITS), 1X linoleic-acid bovine serum albumin (LA-BSA), 10^{-8}M Dexamethasone, 10^{-4}M ascorbic acid 2-phosphate (all from Sigma), 100U penicillin and 1,000U streptomycin (Gibco) with 10 ng/mL vascular endothelial growth factor (VEGF; a kind gift from Dr. S. Ramakrishna, U. of Minnesota). In some instances, fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) was added. Cultures were maintained by media exchange every 4-5 days. Cells were subcultured after day 9 at a 1:4 dilution under the same culture conditions for 20+ population doublings. Most populations used for the vessels have been cultured for more than 5 population doublings.

Medium for Differentiation of Human MAPC Endothelium

For every 100 ml:

Description	Stock Concentration	Final Concentration Amount
VEGF	10 µg/ml	10 ng/ml
Serum-free	N/A	100 ml

For every 100 ml: (serum free medium)

Description	Stock Concentration	Final Concentration	Amount
Dexamethasone	0.25 mM (in H_2O)	0.05 µM	20 µl
ITS	100X	1X	1 ml
Linoleic acid-BSA	100 mg/ml	1 mg/ml	1 ml
DMEM-LG	N/A	N/A	62 ml
MCDB-201	Dissolve in H_2O, adjust pH to 7.1-7.2	Dissolve in H_2O, adjust pH to 7.1-7.2	40 ml
Penicillin/Streptomycin	100X	1X	1 ml
L-Ascorbic Acid	100X (10 mM in PBS)	1X (0.1 mM in PBS)	1 ml

Endothelial differentiation with VEGF was induced by culturing 60-80% (12,500-20,000 cells/cm^2) confluent cultures of MAPCs in serum free medium (58% DMEM-LG, 40% MCDB-201, supplemented with 1X ITS, 1X LA-BSA, 10^{-8}M Dexamethasone, 10^{-4}M ascorbic acid 2-phosphate, 100U penicillin, 1,000U streptomycin) and 10 ng/mL VEGF.

Table 1 summarizes the cell-surface markers expressed on endothelial cells derived from MAPCs and the antibodies used to detect them.

Table 1: Antibodies against cell surface markers expressed on MAPC-derived endothelial cells

Cell Surface Marker	Function	Fluorescent conjugate	Company	Ig Isotype	Flow cytometry
vWF		N/A	Santa Cruz		
β-catenin	Connects to cadherins on cytoskeleton	N/A	Chemicon		
γ-catenin	Connects to cadherins on cytoskeleton	N/A	Chemicon		
Connexin-40	Gap junction protein	N/A	Chemicon		
Connexin-43	Gap junction protein	N/A	Sigma		
ZO-1	Tight junction protein	N/A	Chemicon	IgG	
CD117 (c-Kit)	Transmembrane tyrosine-kinase receptor	R-phycoerythrin	Pharmingen	IgG2b	10 µl/1X10^6
CD62P (p-selectin)	Glycoprotein cell adhesion molecule	FITC	Chemicon	IgG1	
CD62E (e-selectin)	Glycoprotein cell adhesion molecule	FITC	Chemicon	IgG2a	1:20
CD62L (l-selectin)	Glycoprotein cell adhesion molecule	FITC	Sigma	IgG2b	
αVβ3	Vitronectin (integrin) receptor	FITC	Chemicon	IgG1	
αVβ5	Integrin	Phycoerythrin	Chemicon	IgG1	
E-cadherin	Epi. Cell characterization	N/A	Chemicon	IgG1	
VE-cadherin	Cell adhesion molecule	N/A	Chemicon	IgG2a	
Flk1 (VEGF-R2)	Transmembrane tyrosine-kinase receptor	Phycoerythrin	Chemicon	IgG2a	
VCAM-1	Recognizes surface ligand VLA-4	FITC	Chemicon	IgG1	
PECAM-1	Human surface PECAM	FITC	Chemicon	IgG1	
LDL uptake (+)					

Smooth muscle cells can also be induced by culturing MASCs in serum-free medium, without growth factors, supplemented with high concentrations (about 50 to about 200 ng/ml, preferably about ng/ml) of platelet-derived growth factor (PDGF). Cells should preferably be confluent at initiation of differentiation. Terminally differentiated smooth muscle cells can be identified by detecting expression of desmin, smooth muscle specific actin, and smooth muscle specific myosin by standard methods known to those of skill in the art. Smooth muscle actin was detected from day two onwards and smooth muscle myosin after 14 days.

Approximately 70% of cells stained positive with anti-smooth muscle actin and myosin antibodies. A presence of desmin was seen after 6 days. Figure 2 is an immunoblot demonstrating that smooth muscle cell markers α-actin, myosin, desmin, and β-actin (control) are expressed by MAPCs induced with platelet-derived growth factor –BB. Similarly, Figure 3 shows the development of smooth muscle cells from MAPCs cultured in the presence of PDGF-BB. Desmin, smooth muscle actin, and smooth muscle myosin were used as SMC cell surface markers. Immunoglobulin G was used as a negative control.

Example 2: Formation Of a Blood Vessel By Co-Entrapment Of Cells In a Biopolymeric Matrix

In this Example, a blood vessel is engineered by co-entrapment of SMCs and ECs in a fibrin gel tube with VEGF stimulus in the lumen.

Three solutions are required to make a fibrin gel: fibrinogen solution, cell suspension, and thrombin solution. The fibrinogen solution is composed of 5 mg/mL of fibrinogen in 20 mM HEPES buffered saline while the thrombin solution is 150 units of thrombin in 1% water by volume and 9% saline by volume in serum free culture medium with a 0.004 M $CaCl_2$ catalyst. The cell suspension is at six times the final concentration; therefore, the suspension is at 1.5 x 10^6 cell/mL to have a final concentration of 0.25 x 10^6 cells/mL. The fibrinogen solution, the cell suspension, and the thrombin solution are mixed at a ratio of 4:1:1 by volume and gel in less than one hour at 37°C.

The mold to form a tubular fibrin gel for co-culturing cells is composed of a tubular porous polyethylene mandrel (70 μm pore size), rubber stoppers, and a tubular outer casing as shown in Figure 5. Rubber stoppers are placed on the ends of the porous mandrel. The mandrel with stoppers is dipped into a 2% agarose solution to provide a nonstick surface on the outer surface of the polyethylene mandrel. The coated mandrel with stoppers is then placed into an outer casing made of plastic or glass. A needle is inserted between the rubber stopper and the outer casing at one end to allow air to escape as the space is filled with solution. Once the fibrinogen solution, cell suspension, and thrombin solution are mixed, they are injected between the outer casing and the porous mandrel using a needle and syringe. The needles are removed and the solution is then allowed to gel at 37°C in an incubator. A plunger is used to gently push the rubber stoppers, porous mandrel, and fibrin

gel out of the outer casing and into a container containing culture medium. See Figure 4 for a schematic representation of the co-entrapment and organization process.

Rat neonatal smooth muscles (r-neo-SMCs), at a final concentration of 0.25 x 10^6 cells/mL, human multipotent adult progenitor cell-derived endothelial cells (h-MAPC-ECs), at a final concentration of 0.05 x 10^6 cells/mL, and human multipotent adult progenitor cell-derived smooth muscle cells (h-MAPC-SMCs), at a final concentration of 0.05 x 10^6 cells/mL, were used to make sixteen constructs. Two constructs contained r-neo-SMCs and were given TGF-β culture medium, while two constructs contained r-neo-SMCs and h-MAPC-ECs and were given TGF-β culture medium. Two constructs containing h-MAPC-SMCs were given TGF-β medium. Four constructs containing r-neo-SMCs were given both TGF-β and VEGF culture medium, while four constructs containing r-neo-SMC and h-MAPC-ECs were given both TGF-β and VEGF culture medium. Two constructs containing h-MAPC-EC were given both TGF-β and VEGF culture medium. The constructs are summarized below:

No. of Constructs	Cell Type(s)	Culture Medium
2	r-neo-SMC	TGF-β
2	r-neo-SMC and h-MAPC-EC	TGF-β
2	h-MAPC-SMC	TGF-β
4	r-neo-SMC	TGF-β and VEGF
4	r-neo-SMC and h-MAPC-EC	TGF-β and VEGF
2	h-MAPC-EC	TGF-β and VEGF

The TGF-β culture medium consisted of high glucose DMEM, 10% fetal bovine serum, 2 mg/mL amino caproic acid, 2 μg/mL insulin, and 2.5 ng/mL transforming growth factor β (TGF-β). The VEGF culture medium consisted of high glucose DMEM, 10% fetal bovine serum, 2 mg/mL amino caproic acid, 2 μg/mL insulin, and either 50 ng/mL of vascular endothelial growth factor (VEGF) for the first week of culture or 5 ng/mL of VEGF for subsequent weeks. For the final night of culture for all samples, a medium containing high glucose DMEM, 10% fetal bovine serum, 2 mg/mL amino caproic acid, 2 μg/mL insulin, and 10 μg/mL Dil-Ac-LDL was used to assist in immunostaining.

The top stoppers were removed after the samples were ejected from the mold, and the culture medium was changed three times a week with 5-mL (approximately three-fourths of the total volume of medium) of the TGF-β culture medium being removed and replaced each

time. For samples containing endothelial cells, 0.5 mL of the VEGF culture medium (at concentrations 50 ng/mL for the first week and 5 ng/mL for subsequent weeks) was injected into the lumen of the porous mandrel with a syringe at each changing of the culture medium. All samples were cultured in a 5% CO_2 incubator. Sterile forceps were used to loosen the gel from the ends of the mandrel as necessary during the first week to allow compaction of the gel to occur.

The dimensions of the molds used to make the samples were a 0.25 inch outer diameter, 0.156 inch inner diameter, 2 inch long 70 μm porous hollow polyethylene mandrel; two rubber plunger tips from 3cc syringes inside two rubber plunger tips from 6cc syringes; and a plastic cylinder longer than 2 inches from a 6cc syringe.

After one week, one construct with r-neo-SMCs receiving TGF-β and VEGF culture mediums as well as one construct with r-neo-SMCs and h-MAPC-ECs receiving TGF-β and VEGF culture media were harvested and frozen in OCT for frozen tissue sectioning. After two weeks, one construct with r-neo-SMCs receiving TGF-β and VEGF culture media and h-MAPC-ECs receiving TGF-β and VEGF culture media were harvested and frozen in OCT for frozen tissue sectioning. After four weeks, all remaining samples were harvested and frozen in OCT for frozen tissue sectioning.

Tissues were sectioned into 10 μm slices. Masson's Trichrome staining (Figure 6) and Verhoeff's Van Gieson staining (Figure 7) was used to assess extracellular matrix production, and immunohistological staining was performed to detect vWF, LDL, collagen type IV, smooth muscle actin, and DAPI (4,6-diaminophenylindole) to ascertain the relative location of the endothelial cells and the smooth muscle cells.

Example 3: Characterization Of Layered Circumferential Vascular Structure

After culturing the constructs from Example 2 on the agarose-coated porous polyethylene tube for 7 days, ECs were mainly located close to the lumenal surface (Figure 8), however the cells were still somewhat disorganized, as seen in Figure 9. After 3 weeks of culture, the cells began to align and form prototypical layers, as detected by vWF staining, LDL uptake, and DAPI staining (Figure 10). ECs were seen close to the lumenal surface. Figure 11A show a cross-section of the entire construct at lower magnification immunostained for α-smooth muscle-actin (red), collagen type IV (green), and DAPI (blue).

SMCs populated the interior of the construct and ECs were absent from the outer surface, which was surrounded by medium that was not supplemented with VEGF, indicating that ECs can be selectively localized to surfaces after co-entrapment by presenting a concentration gradient of VEGF, presumably by a chemotactic response. Figures 11B and C show marked organization of the cellular layers, especially of a section near the lumenal surface of the construct after 5 weeks of incubation. The vessel was immunostained for low-density lipoprotein (red), collagen type IV (green), and DAPI (purple – stains cell nuclei).

In summary, endothelial cells localized to the lumenal surface of the tubular constructs, which was the surface in contact with the VEGF-supplemented medium. As the SMCs commenced to remodel the fibrin into cell-derived ECM, the nascent endothelium matured. Additionally, type IV collagen staining shows the presence of basement membrane formation between the two cell layers. Formation of the basement membrane should confer endothelial stability and paracrine factor effects that lead to a quiescent endothelium as well as improved ECM formation by the tissue cells (and hence better construct properties).

Table 2 is a summary of the expression pattern of cell surface markers found on endothelial cells derived from MAPCs.

Table 2: Expression profile for cell surface molecules of endothelium

Endothelial cells were replated at a cell density of 7000 – 9000 cells/cm^2.

Cell Surface Markers	Days Expressed	Working Dilutions
Flk1	Low to d9 – d18	1:50
Flt1	Low to d9 – d18	1:50
VE-cadherin	d3 – d18	1:50
AC133	Low before d3	1:50
αVβ5	d3 – d14	1:50
αVβ3	d3 – d14	1:50
Tie	After d7	1:50
Tek	After d3	1:50
vWF	After d9	1:50
CD31	> d14	1:50
CD36	> d14	1:50
CD62-P	> d14	1:50
ZO-1	> d14	1:50
β-catenin	> d14	1:50
γ-catenin	> d14	1:50

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Although the foregoing invention has been described in some detail by way of illustrations and examples for purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention.

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The invention is further described by the following numbered paragraphs:

1. A method of producing a tissue-engineered blood vessel, comprising:

a. entrapping at least endothelial cells (ECs) and smooth muscle cells (SMCs) in a biopolymeric matrix; and

b. culturing the cells on the outer surface of a porous tube, wherein medium comprising a tactic factor is present inside the tube, such that a bilayered structure is formed;

thereby producing a tissue-engineered blood vessel.

2. The method of paragraph 1, wherein the ECs and/or the SMCs are derived from stem cells.

3. The method of paragraph 2, wherein the stem cells are selected from the group consisting of embryonic stem (ES) cells, embryonic germ (EG) cells, multipotent adult progenitor cells (MAPCs), hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and endothelial progenitor cells (EPCs).

4. The method of paragraph 3, wherein the stem cells are MAPCs.

5. The method of paragraph 2, wherein the stem cells are autologous.

6. The method of paragraph 2, wherein the stem cells are heterologous.

7. The method of paragraph 1, wherein the ECs and/or the SMCs are derived from autologous vascular tissue.

8. The method of paragraph 1, wherein the ECs and/or the SMCs are derived from heterologous vascular tissue.

9. The method of paragraph 7, wherein the autologous vascular tissue is selected from the group consisting of pulmonary artery, pulmonary vein, femoral artery, femoral vein, saphenous artery, saphenous vein, iliac artery, iliac vein, umbilical artery, umbilical vein, microvascular tissue, and aortic tissue.

10. The method of paragraph 8, wherein the heterologous vascular tissue is selected from the group consisting of pulmonary artery, pulmonary vein, femoral artery, femoral vein, saphenous artery, saphenous vein, iliac artery, iliac vein, umbilical artery, umbilical vein, microvascular tissue, and aortic tissue.

11. The method of paragraph 9 or 10, wherein the microvascular tissue is derived from heart, lung, liver, kidney, brain or dermal tissue.

12. The method of paragraph 2, wherein the stem cells are derived from bone marrow, brain, spinal cord, umbilical cord blood, liver, muscle, fat or placenta.

13. The method of paragraph 1, wherein the biopolymeric matrix is selected from the group consisting of fibrin, fibrinogen and thrombin, laminin, collagen, proteoglycans, amphiphilic di-block copolymers, and amphiphilic tri-block copolymers.

14. The method of paragraph 13, wherein the biopolymeric matrix is fibrin.

15. The method of paragraph 1, wherein the tube is comprised of porous plastic.

16. The method of paragraph 15, wherein the porous plastic is polyethylene.

17. The method of paragraph 15, wherein the tube further comprises agarose.

18. The method of paragraph 1, wherein tissue types of the tissue-engineered blood vessel are identified by immunostaining, immunoblotting, magnetic beads, flow cytometry, microarray analysis, or RT-PCR.

19. The method of paragraph 1, wherein the bilayered structure comprises a medial layer and an intimal layer.

20. The method of paragraph 19, wherein the bilayered structure further comprises a basement membrane and a lumen.

21. The method of paragraph 1, wherein the tactic factor is vascular endothelial growth factor (VEGF).

22. A method of providing a tissue-engineered vascular graft to a subject in need thereof, comprising:

(a) entrapping at least endothelial cells (ECs) and smooth muscle cells (SMCs) in a biopolymeric matrix;

(b) culturing the cells on the outer surface of a tube, wherein medium comprising a tactic factor is present inside the tube, such that a bilayered structure is formed, thereby producing a tissue-engineered blood vessel; and

(c) implanting the tissue-engineered blood vessel into the subject.

23. The method of paragraph 22, wherein the subject is a human.

24. The method of paragraph 22, wherein the tissue-engineered blood vessel is vasoactive.

25. The method of paragraph 22, wherein the ECs and/or the SMCs are derived from stem cells.

26. The method of paragraph 25, wherein the stem cells are selected from the group consisting of embryonic stem (ES) cells, embryonic germ (EG) cells, multipotent adult progenitor cells (MAPCs), hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and endothelial progenitor cells (EPCs).

27. The method of paragraph 26, wherein the stem cells are MAPCs.

28. The method of paragraph 25, wherein the stem cells are autologous.

29. The method of paragraph 25, wherein the stem cells are heterologous.

30. The method of paragraph 22, wherein the ECs and/or the SMCs are derived from autologous vascular tissue.

31. The method of paragraph 22, wherein the ECs and/or the SMCs are derived from heterologous vascular tissue.

32. The method of paragraph 30, wherein the autologous vascular tissue is selected from the group consisting of pulmonary artery, pulmonary vein, femoral artery, femoral vein, saphenous artery, saphenous vein, iliac artery, iliac vein, umbilical artery, umbilical vein, microvascular tissue, and aortic tissue.

33. The method of paragraph 31, wherein the heterologous vascular tissue is selected from the group consisting of pulmonary artery, pulmonary vein, femoral artery, femoral vein, saphenous artery, saphenous vein, iliac artery, iliac vein, umbilical artery, umbilical vein, microvascular tissue, and aortic tissue.

34. The method of paragraph 32 or 33, wherein the microvascular tissue is derived from heart, lung, liver, kidney, brain or dermal tissue.

35. The method of paragraph 25, wherein the stem cells are derived from bone marrow, brain, spinal cord, umbilical cord blood, liver, muscle, fat or placenta.

36. The method of paragraph 22, wherein the biopolymeric matrix is selected from the group consisting of fibrin, fibrinogen and thrombin, laminin, collagen, proteoglycans, amphiphilic di-block copolymers, and amphiphilic tri-block copolymers.

37. The method of paragraph 36, wherein the biopolymeric matrix is fibrin.

38. The method of paragraph 22, wherein the tactic factor is vascular endothelial growth factor (VEGF).

39. The method of paragraph 22, wherein the tube is comprised of porous plastic.

40. The method of paragraph 39, wherein the porous plastic is polyethylene.

41. The method of paragraph 40, wherein the tube further comprises agarose.

42. The method of paragraph 23, wherein tissue types of the tissue-engineered blood vessel are identified by immunostaining, immunoblotting, magnetic beads, flow cytometry, microarray analysis, or RT-PCR.

43. The method of paragraph 22, wherein the bilayered structure comprises a medial layer and an intimal layer.

44. The method of paragraph 43, wherein the bilayered structure further comprises a basement membrane and a lumen.

45. A cell-matrix structure for use as a vascular tissue comprising a medial layer of cells and an intimal layer of cells.

46. The cell-matrix structure of paragraph 45, further comprising a basement membrane and a lumen.

47. The cell-matrix structure of paragraph 45, wherein the structure is implantable.

48. The cell-matrix structure of paragraph 45, wherein the medial layer comprises SMCs.

49. The cell-matrix structure of paragraph 45, wherein the intimal layer comprises ECs.

50. The cell-matrix structure of paragraph 45, wherein the matrix is comprised of a biocompatible polymer.

51. The cell-matrix structure of paragraph 45, wherein the matrix is biodegradable.

52. The cell-matrix structure of paragraph 50, wherein the matrix is selected from the group consisting of fibrin, fibrinogen and thrombin, laminin, collagen, proteoglycans, amphiphilic di-block copolymers, and amphiphilic tri-block copolymers.

53. The cell-matrix structure of paragraph 52, wherein the matrix is fibrin.

54. The cell-matrix structure of paragraph 45, wherein the vascular tissue is an artery.

55. The cell-matrix structure of paragraph 45, wherein the vascular tissue is a vein.

56. The cell-matrix structure of paragraph 45, wherein the vascular tissue is vasoactive.

57. The cell-matrix structure of paragraph 45, wherein the structure is non-immunogenic when implanted in a subject.

58. The cell-matrix structure of paragraph 45, wherein the structure is in culture and wherein the intimal layer is in contact with a medium comprising VEGF.

59. The cell-matrix structure of paragraph 45, further comprising a porous hollow tube, wherein the intimal layer of cells and the medial layer of cells are positioned concentrically around the tube.

60. The cell-matrix structure of paragraph 59, further comprising an outer casing surrounding the cell-matrix structure.

61. The method of paragraph 1, further comprising:

c. coating the bilayered structure with fibroblasts to form a trilayered structure.

62. The method of paragraph 22, further comprising the step of coating the bilayered structure with fibroblasts to form a trilayered structure after step (b) and before step (c).

63. The method of paragraph 61, wherein the fibroblasts are derived from stem cells.

64. The method of paragraph 63, wherein the stem cells are selected from the group consisting of embryonic stem (ES) cells, embryonic germ (EG) cells, multipotent adult progenitor cells (MAPCs), hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and endothelial progenitor cells (EPCs).

65. The method of paragraph 63, wherein the stem cells are MAPCs.

66. A tissue-engineered-blood vessel produced by the method of paragraph 2.

67. A tissue-engineered-blood vessel produced by the method of paragraph 4.

68. A tissue-engineered blood vessel produced by the method of paragraph 61.

69. A tissue-engineered blood vessel produced by the method of paragraph 63.

70. A method of culturing cells comprising endothelial cells (ECs) and smooth muscle cells (SMCs) entrapped in a biopolymeric matrix comprising the steps of growing the ECs and SMCs on the exterior surface of a porous hollow tube and providing medium comprising tactic factors axially within the interior of the porous hollow tube, such that a

bilayered hollow tubular structure of cells comprising endothelial cells (ECs) positioned interiorly and smooth muscle cells (SMCs) positioned exteriorly is formed, thereby producing a tissue-engineered blood vessel.

71. The method of paragraph 70, wherein an outer casing surrounds the bilayered hollow tubular structure.

72. The method of paragraph 70, wherein the medium comprising tactic factors is flowing through the porous hollow tube.

ABSTRACT OF THE INVENTION

Provided are a tissue-engineered blood vessel and methods for producing tissue-engineered blood vessels by entrapping at least endothelial cells (ECs) and smooth muscle cells (SMCs) in a biopolymeric matrix; and culturing the cells on the outer surface of a porous tube, wherein medium comprising a tactic factor is present inside the tube, such that a bilayered structure is formed.

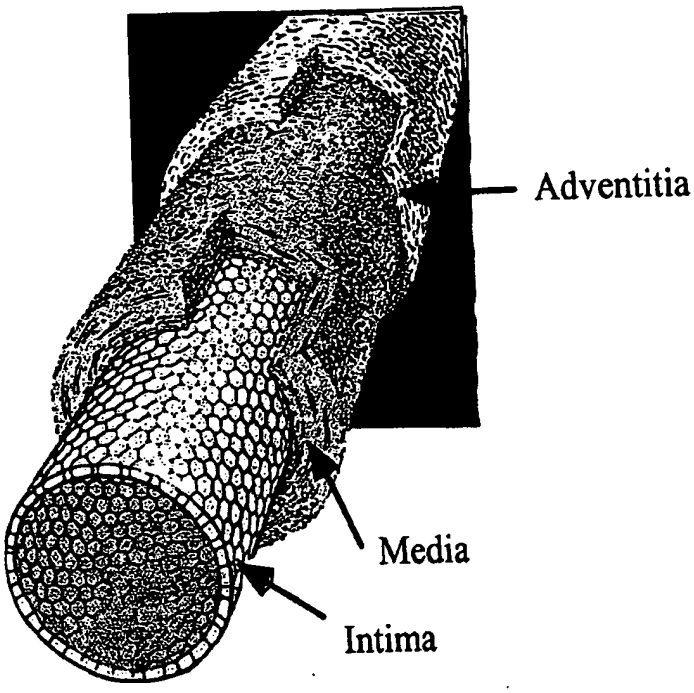

Figure 1

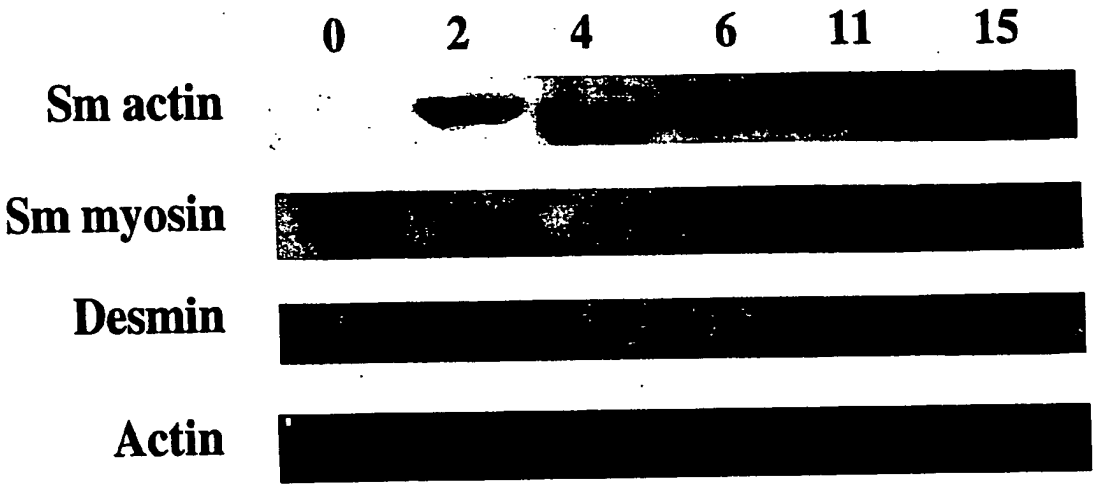

Figure 2

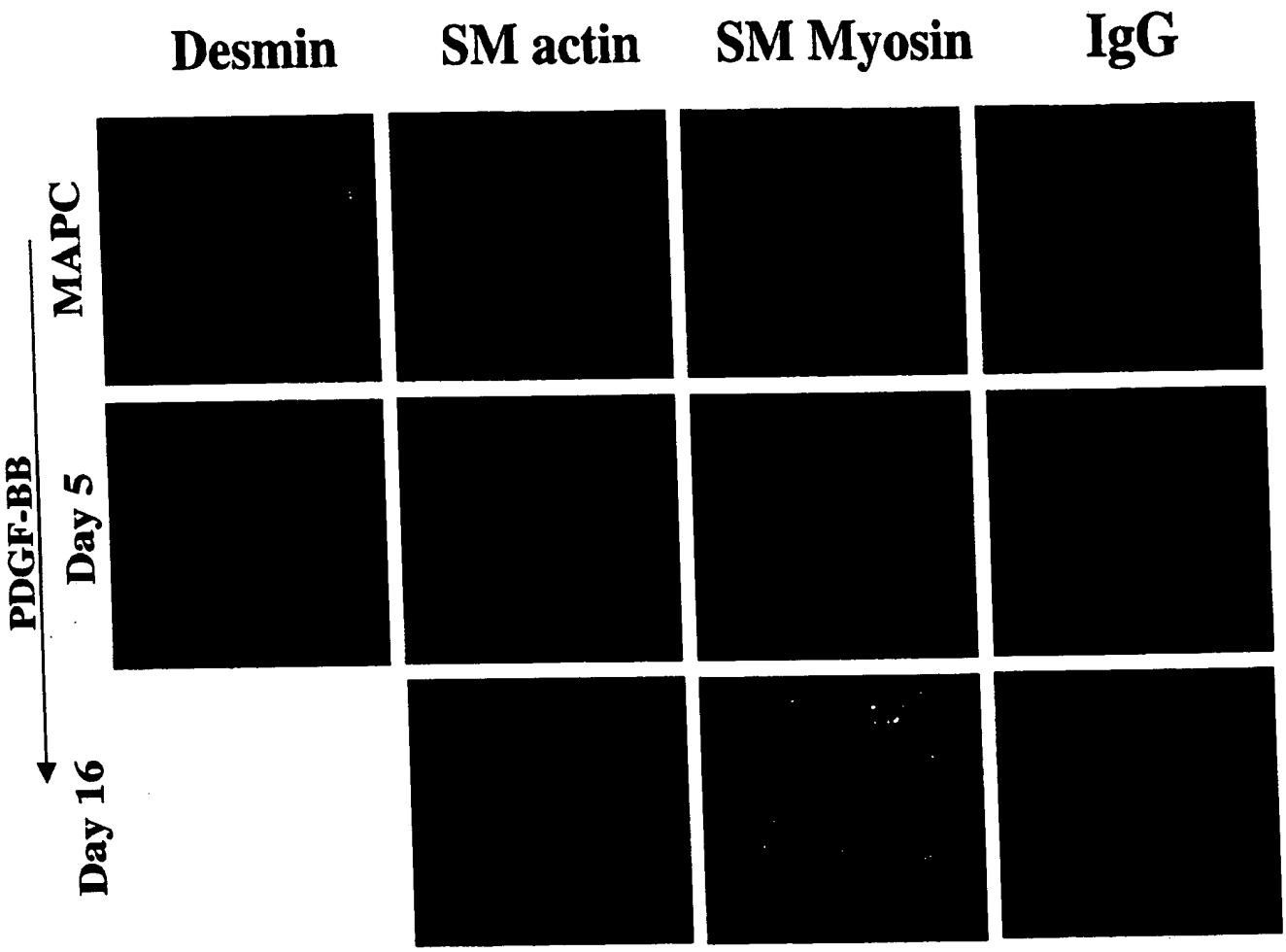

Figure 3

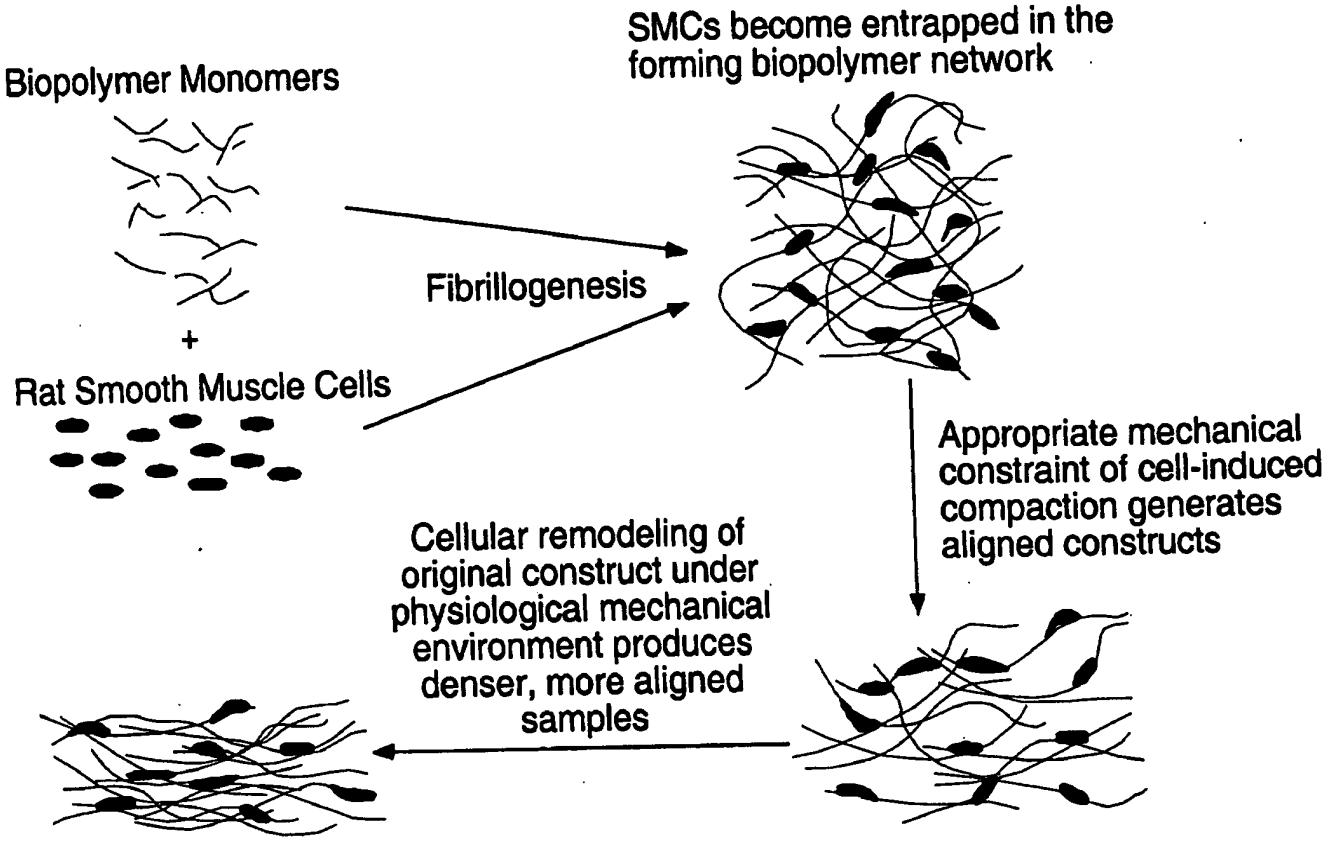
Biopolymer Monomers
+
Rat Smooth Muscle Cells
Fibrillogenesis
SMCs become entrapped in the forming biopolymer network
Appropriate mechanical constraint of cell-induced compaction generates aligned constructs
Cellular remodeling of original construct under physiological mechanical environment produces denser, more aligned samples

Figure 4

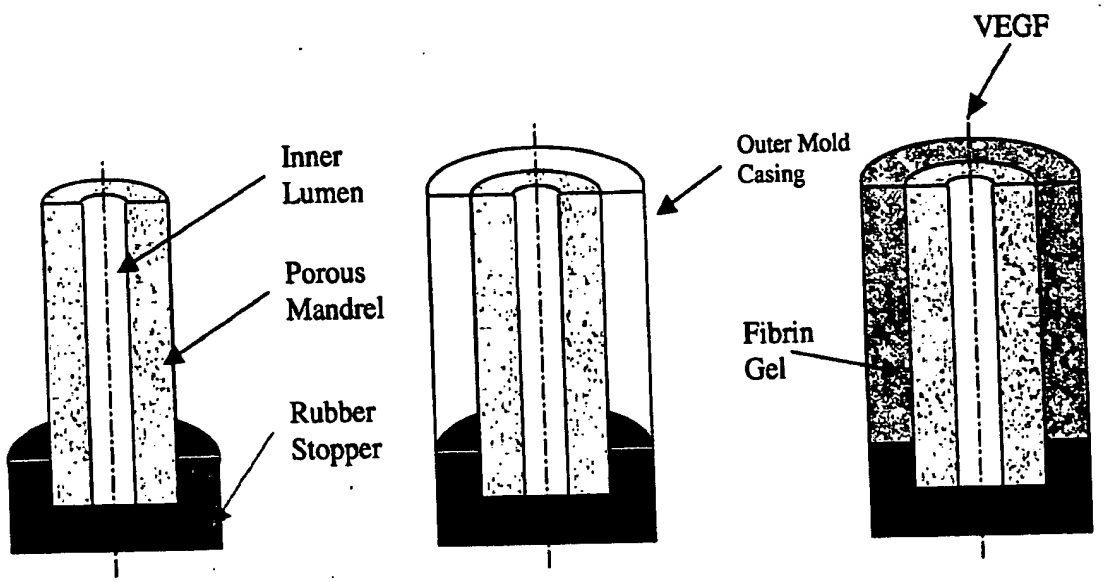
Inner
Lumen
Porous
Mandrel
Rubber
Stopper
Outer Mold
Casing
VEGF
Fibrin
Gel

Figure 5

Figure 6

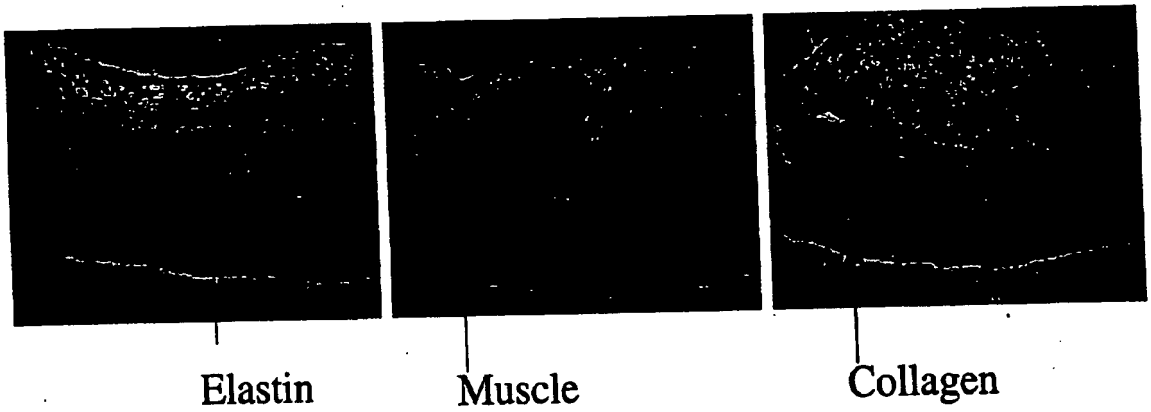

Figure 7

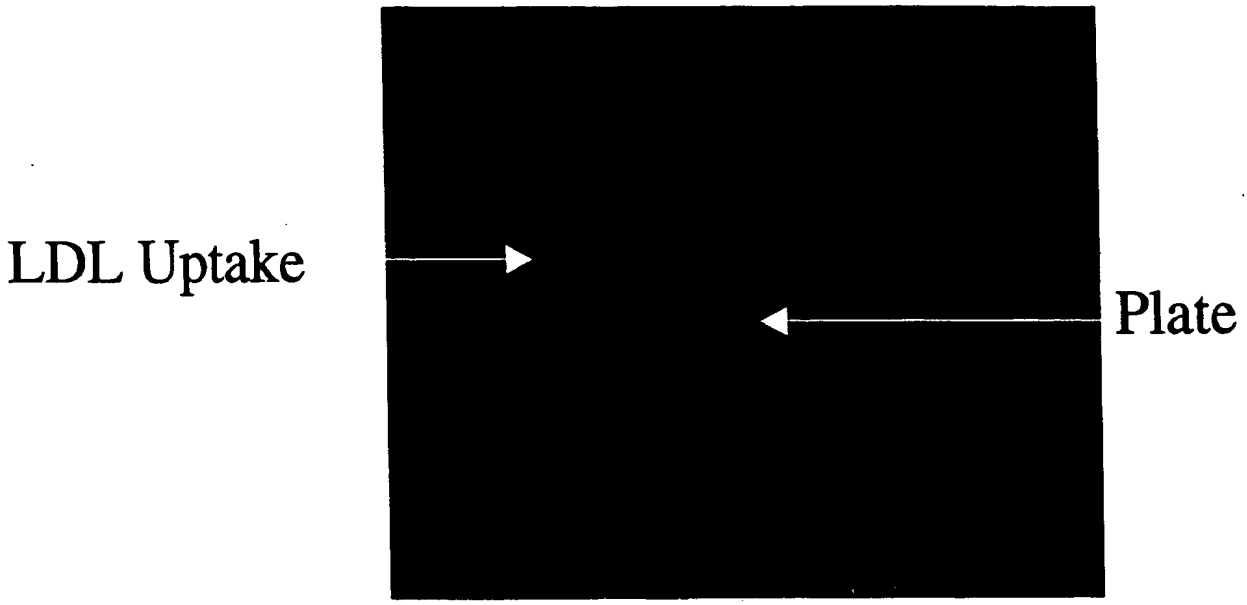

Figure 8

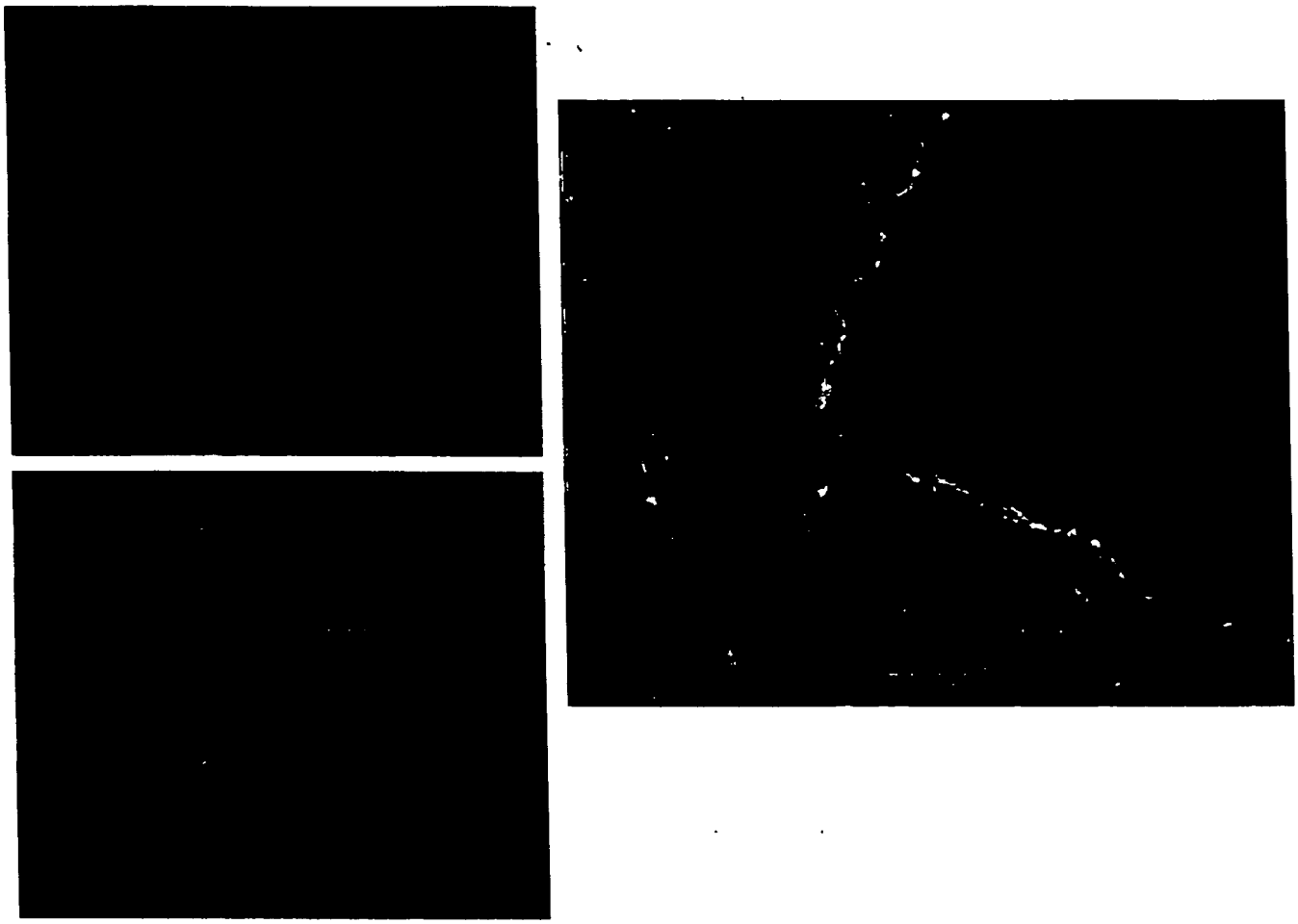

Figure 9

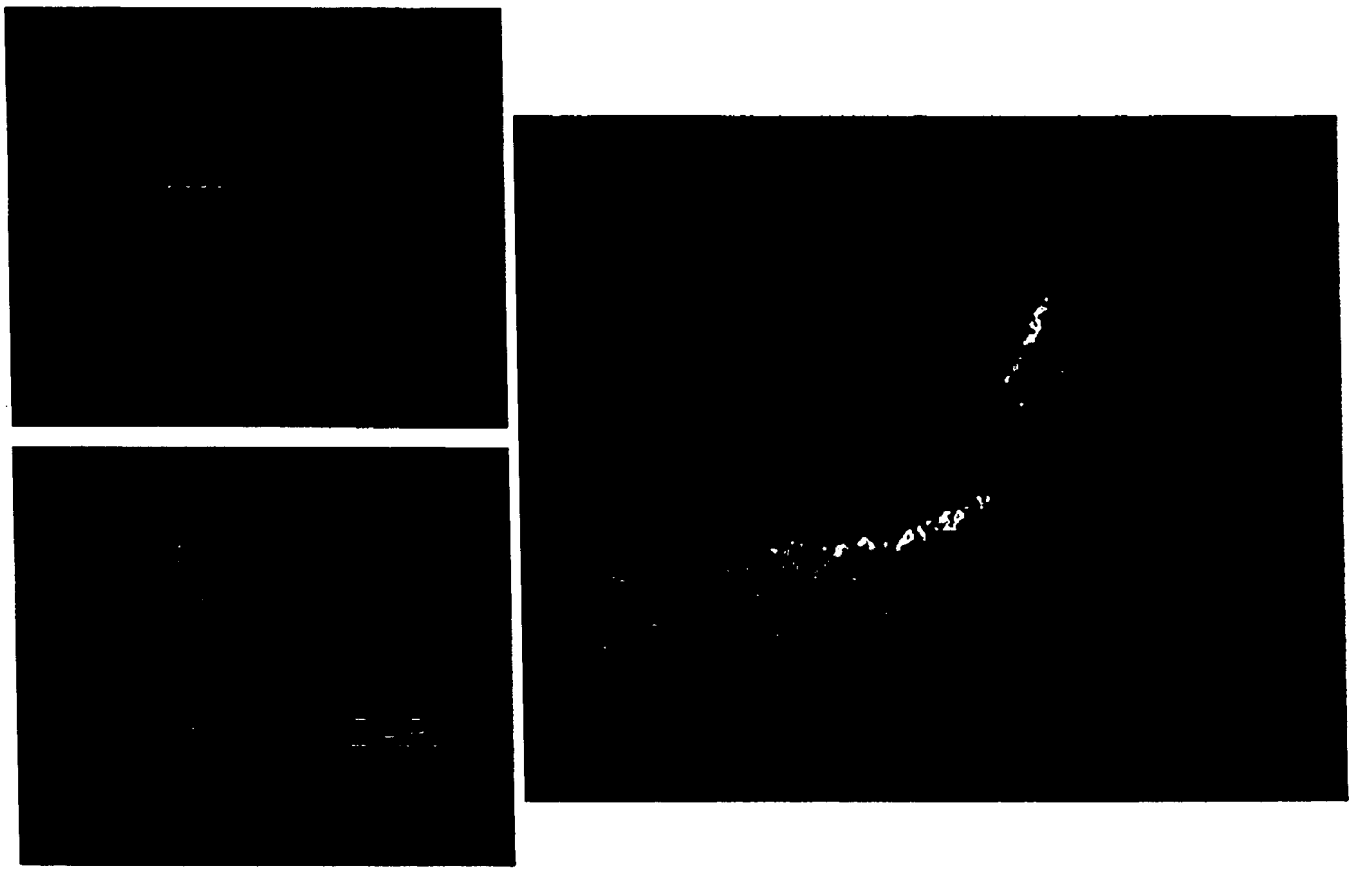

Figure 10

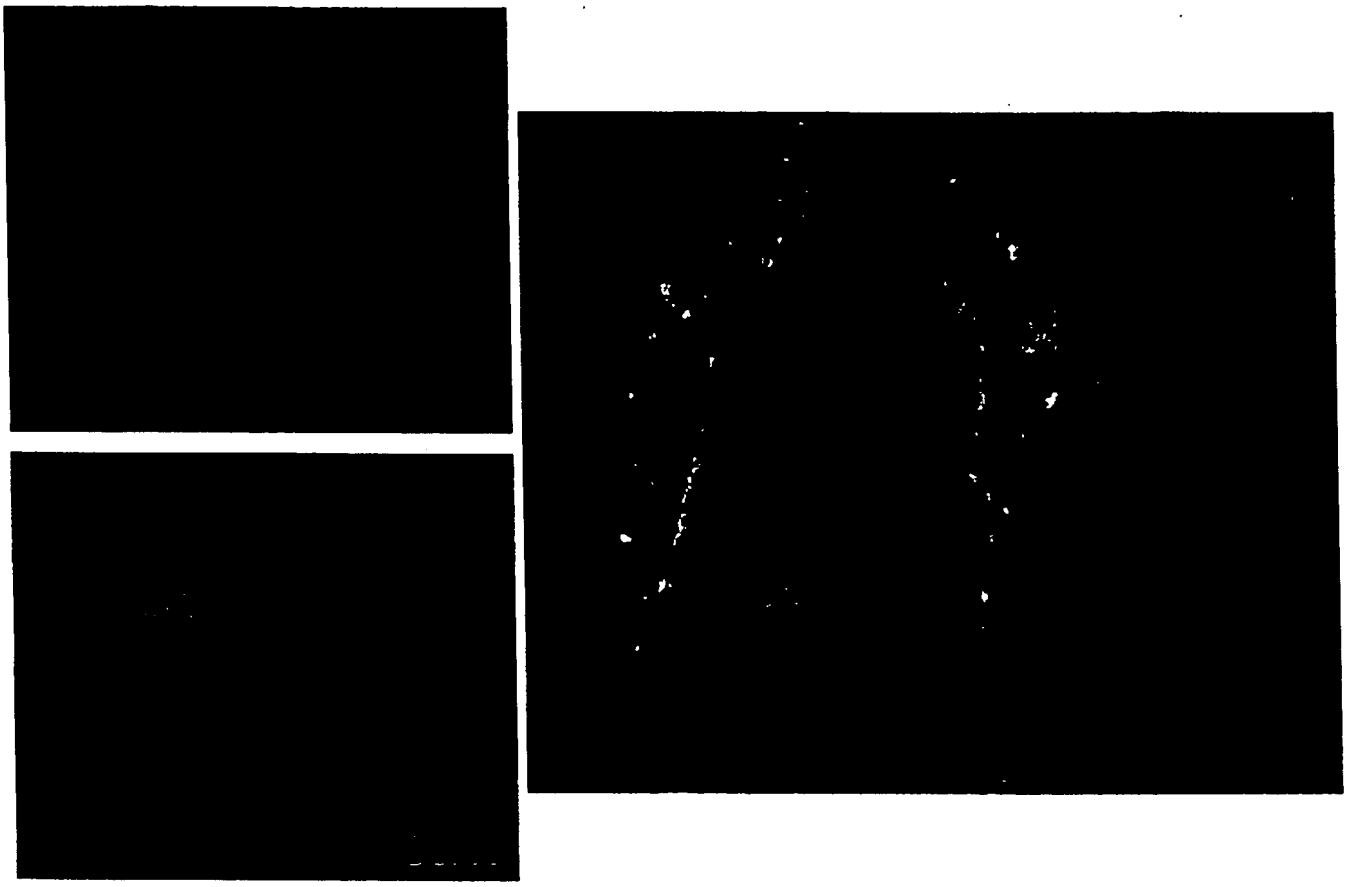

Figure 11A

Figure 11B

Figure 11C